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Evidence for a Flowering Inhibitor Produced in Long Days in *Kalanchoe blossfeldiana*

BY

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With three Figures in the Text

Abstract

Experiments with *Kalanchoe blossfeldiana* are described in which periods of short-day treatment were interrupted by intercalated long days or light breaks during long dark periods. The effects of 24-hour dark periods preceding and following such intercalated long days were also investigated.

The results of these experiments have shown that: Single long days intercalated between numbers of short days have a positive inhibitory effect on flower initiation and are not merely ineffective. The inhibitory effect expressed as the number of inductive cycles annulled is approximately additive, provided the long days are interspersed with short days, but not if several long days are given consecutively. On the average 1 long day is capable of annulling the flower-promoting effect of about 1½–2 short days. To a first approximation flower numbers in *Kalanchoe* increase exponentially with the number of inductive cycles given—up to at least 12 short days; the inhibitory effect of long days interspersed with short days also fits an exponential curve; i.e. the inhibition is roughly proportional to the amount of previous photo-periodic induction. A light break of as little as 30 seconds' duration given in the middle of a long dark period is as inhibitory as a long day. If followed by a long dark period the inhibition of an intercalated long day is almost completely neutralized; a long dark period preceding it has no such effect.

These results have been interpreted as due to the interaction of a flowering inhibitor with a reaction leading to flowering. A mechanism involving competitive inhibition of an adaptively formed enzyme has been described as a possible example of the kind of reaction which could account for the results presented.

Introduction

IN the study of daylength effects on the flowering of plants it has been known for a long time that a series of inductive cycles must be given in order to promote flowering. Even in *Xanthium pennsylvanicum* in which a single long night will lead to flowering if preceded by an adequate light period (Naylor, 1941) the response is more rapid, and more female inflorescences are produced if several inductive cycles are given. This is clearly due to a summation of the effects of favourable cycles. If the plant has received a sufficient number of these, progress towards flowering will be maintained even if the plant is placed into daylength conditions unfavourable for starting the process. This is the so-called 'photoperiodic after-effect'.

It is equally well known that in many short-day plants such summation will

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not occur when the favourable inductive cycles are not given in succession but are separated by numbers of non-inductive cycles (Long, 1939). This also seemed to constitute a qualitative difference from long-day plants in which summation of separate inductive cycles does occur. However, according to Melchers (1952) Carr has obtained evidence that in some instances at least the difference is only quantitative.

In nearly all existing 'theories' of the mechanism of photoperiodism each complete cycle of light and darkness is regarded as a unit whose effect is independent of preceding or following cycles, e.g. Gregory (1948), Hamner (1940), Harder and Bode (1943), Bünning (1950), though accumulation of a flowering substance over a number of favourable cycles, up to a critical level, had been foreseen, after which floral initiation would take place. In these theoretical discussions scant attention has been paid to the fact that accumulation can occur in short-day plants if favourable cycles follow one another, but not if long-day periods are interposed. The experiments to be described represent an attempt to investigate this aspect from a quantitative point of view.

Methods and Results

Plants of *Kalanchoe blossfeldiana* were raised from cuttings in continuous light (natural day plus incandescent light). After the actual experimental treatments they were again placed into continuous light or, where stated, into a long day consisting of 8 hours' daylight followed by 8 hours' incandescent light and 8 hours' dark. In all the experiments unless otherwise stated the total cycle length amounted to 24 hours.

The data collected included: the number of days from the beginning of the experiment to the macroscopic appearance of the inflorescence, the number of flowers produced, the leaf number, and height increments of the plants.

In a preliminary experiment a total of 12 short days was given in every treatment, each of these being separated from the next by a certain number of long days; in the various treatments the numbers of these intercalated long days ranged from 1 to 25. The result of this experiment was entirely negative; only the controls with no intercalated long days flowered. Even a single long day between each pair of short days led to complete suppression of flower initiation.

In the next experiment 12 short-day inductive cycles were again given which were split into groups always separated by a single long day. The six treatments were: (1) one group of 12 short days (control), (2) two groups of 6 short days, (3) three groups of 4 short days, (4) four groups of 3 short days, (5) six groups of 2 short days, and (6) alternating short and long days until a total of 12 short days had been given. In this experiment the long days consisted of 8 hours' daylight extended to 16½ hours' light by incandescent lamps. Before and after treatment the plants were also kept in a 16½-hour day. Twelve replicates were used in each treatment. The mean number of flowers produced

per plant in each of these treatments is shown in Table I; in the bottom line they are shown as percentages of the control.

These results indicate the magnitude of the unfavourable effect of increasing numbers of intercalated long-day cycles on flower numbers, e.g. a single long-day cycle given between two groups of 6 short days is sufficient to reduce the degree of flowering to half that in the controls. Alternation of long and short days again caused complete failure to flower.

TABLE I

Effect of Interrupting a Period of Twelve Short-day Cycles by Single Long-day Cycles on the Flowering of Kalanchoe. Twelve Replicates per Treatment

Number of S.D. cycles given consecutively .	12	6	4	3	2	1
Number of groups of such cycles always separated by 1 long day . . .	1	2	3	4	6	12
Number of plants budded . . .	12	12	11	6	7	0
Number of flowers per plant . . .	64·8	33·2	7·6	1·8	1·8	0
Number of flowers as per cent. of control .	100	51·2	11·7	2·8	2·8	0

In another experiment, designed to estimate quantitatively the effect of the duration of a light break in the middle of a long dark period, again a period of 12 short days was given which was split up into 3 groups of 4 short days each. These were separated not by long days but by a short photoperiod and an interrupted long dark period; i.e. during a total period of treatment of 14 days light breaks were given on two occasions: in the middle of the fifth and tenth

TABLE II

Effect of Duration of Light Break given in the Middle of the Fifth and Tenth Nights of a Period of Fourteen Short Days (see text). Ten Replicates per Treatment

Duration of light break . .	0 (control)	30 sec.	2 min.	7½ min.	½ hr.	2 hr.
Number of plants budded . .	10	5	4	8	7	4
Number of flowers per plant .	111·0	10·9	1·0	3·6	19·0	1·1

nights. The treatments were: Control (no light breaks), 12 consecutive short days; light breaks in the fifth and tenth nights of the following duration—30 seconds, 2 minutes, 7½ minutes, 30 minutes, and 2 hours. Ten replicates were used in each of the six treatments. The light intensity of the interrupting light exceeded 1,500 ft. c. The numbers of plants budded and the number of flowers per plant are shown in Table II.

There appear to be no significant differences between any of the light break treatments, and 30 seconds of bright light were about as effective as 2 hours in annulling a large amount of the induction due to the twelve uninterrupted short days given. Thus two 30-second light breaks given at 4-day intervals reduced flowering by 90 per cent. compared with the controls.

Taken together it is quite obvious from these two experiments that intercalated long days or light breaks have a much larger annulling effect than

mere destruction of the flower-promoting effect of the actual cycle they are given in.

A quantitative estimate of the negative effect was attempted in the following experiment. Apart from repeating the treatments of intercalating 1, 2, 3, and 5 long days symmetrically between a constant number of 12 short days, another group of plants was given different numbers of inductive cycles, i.e. 12, 6, 4, 3, and 2 short days preceded and followed by continuous light. The numbers of plants budded in the different treatments and the mean numbers of flowers per plant are recorded in Table III. The mean number of days from the beginning of the experiment to the macroscopic appearance of the inflorescence are also shown.

TABLE III

Effect of Inductive Cycles on the Degree of Flowering in Kalanchoe in Comparison with the Annulling Effect of Intercalated Long-day Cycles. Ten Replicates per Treatment

Number of inductive cycles	Number of plants budded	Mean number of flowers per plant	Mean number of days to budding (excl. vegetative plants)
12	10	234·5	19·9
6	10	14·1	32·4
4	7	0·8	38·1
3	3	0·6	33·7
2	1	0·1	25
Number of intercalated long days in a series of 12 short days			
1	10	134·5	20·6
2	10	109·7	26·0
3	9	8·7	29·1
5	6	1·6	31·5

As expected flower numbers increase rapidly with increasing numbers of inductive cycles, though the acceleration seems very high. Also the number of days to budding falls (at least over the range of 4–12 cycles in which most plants budded) with increasing induction. Those treatments with intercalated long days confirm the results of the experiment shown in Table I.

In Fig. 1 the logarithms of the flower numbers of the treatments having received different induction periods have been plotted against the number of days of induction and an approximately straight line relation seems to obtain. A regression line significant at the 0·01 per cent. level has been fitted to these data and is also shown. Now, by determining on this line the position of the treatments with intercalated long days we can estimate to what uninterrupted induction period each of these corresponds, and hence how much of the effect of the constant number of 12 short days has been annulled. This has been done, and from the figures obtained we can calculate the average negative effect for a single interposed long day as shown in Table IV.

On the basis of flower numbers the mean annulling effect of 1 long day

amounts therefore to the destruction of 1·4 short days—quite apart from being itself non-inductive, i.e. 2·4 cycles are eliminated. When more than 1 long day is intercalated a corresponding number of short days is annulled and the

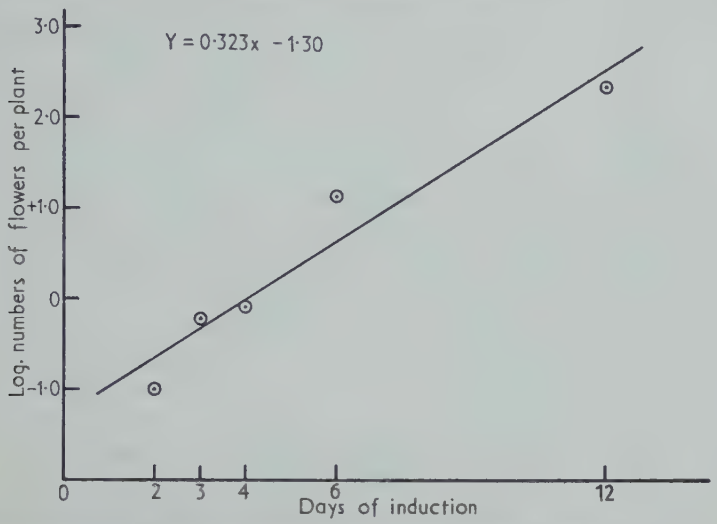

FIG. 1. Relation between the number of inductive cycles and the logarithm of flower numbers. First experiment.

deviations from this mean are not large (Table IV). It should be noted here that this relation was established on the basis of the logarithm of flower numbers and thus the effect of each long day is proportional to the degree of induction.

TABLE IV

Mean Annulling Effect of Intercalated Long Days

	On the basis of log. flower numbers			On the basis of the reciprocal of the number of days to budding		
Number of long days interrupting 12 short days symmetrically	Corresponding number of days of uninterrupted induction	Number of short days annulled	Expected mean effect	Corresponding number of days of uninterrupted induction	Number of short days annulled	Expected mean effect
1	10·6	1·4	1·4	11·0	1·0	1·6
2	10·4	1·6	2·8	8·9	3·1	3·1
3	6·9	5·1	4·3	7·4	4·6	4·7
5	4·7	7·3	7·1	3·7	8·3	7·8
Mean per single long day		1·42			1·55	

Using the reciprocal of the number of days to budding—so as to include those plants which remained vegetative as zero values—an exactly similar calculation was made for this characteristic and is shown in Fig. 2. Again a linear regression fitted the data highly significantly. On this basis the average effect of 1 long day amounted to the destruction of 1·55 short days. As will

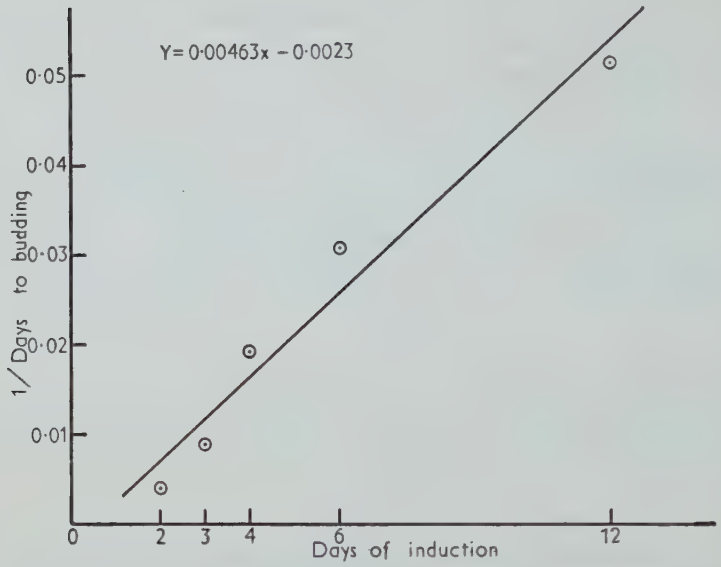

FIG. 2. Relation between the number of inductive cycles and the reciprocal of the numbers of days to the macroscopic appearance of the inflorescence.

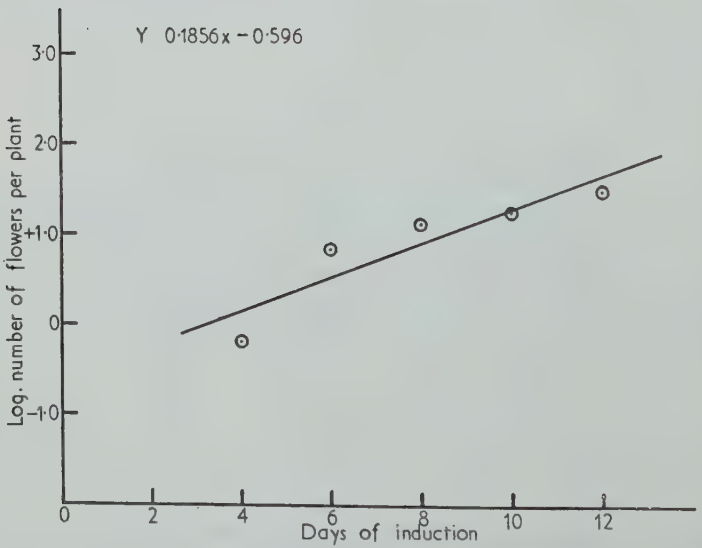

FIG. 3. Relation between the number of inductive cycles and the logarithm of flower numbers. Second experiment.

be seen from the data in Table IV, in this case also the deviations from this mean value were not large. In both these calculations only the long days intercalated between the constant 12 short days have been taken into account, although it would seem very probable that at least the long day preceding or following the total period of short-day treatments will also have had an effect. However, this applies equally to the control treatments without interposed long days and has therefore been left out of account.

In order further to confirm the apparent exponential increase in flower numbers with increasing induction up to at least 12 short days (approximate doubling of flower numbers for each extra short day given) a further experiment was set up. In this 2, 4, 6, 8, 10, and 12 short days were given to groups of 10 replicate plants. In Fig. 3 the log. flower numbers has been plotted against the number of days of induction. At the same time six similar groups of plants were given 12 short days with a single intercalated long day, but the position of this interrupting day was varied. In the different treatments it followed 0, 2, 4, 6, 8, and 10 short days, the total number being made up to 12 in each case by further short days following the interruption. The number of days from the start of the experiment until the macroscopic appearance of the inflorescences and the numbers of flowers produced are shown in Table V.

TABLE V

Effect of the Position of a Single Long Day interrupting a Constant Period of Twelve Short Days. Eight Replicates per Treatment

	Number of short days preceding the interrupting long day:					
	0 (control)	2	4	6	8	10
Number of plants budded . .	8	8	8	8	8	8
Mean number of flowers per plant .	35·0	20·0	11·9	12·9	19·6	23·4
Mean number of days to budding .	16·8	17·8	18·4	18·3	17·6	17·0

The flower number data shown in Table V confirm that a single intercalated long day has a marked effect in reducing flower numbers. This is so in whatever position it occurs among the 12 short days. However, it is also clear that the effect is greatest when the interruption falls near the middle of the run of short days. This was confirmed by the significance of the quadratic term of a regression of log. flower numbers on the number of short days preceding the interruption. The magnitude of the position effect is not very large, however, when compared with the effect of increasing numbers of intercalated long days.

Taking the mean flower number of all treatments with an intercalated long day, the average inhibitory effect, expressed as the number of inductive cycles annulled, was estimated from Fig. 3 in the same manner as shown in Table IV above. In this experiment the value obtained was: 2·1 short days annulled for 1 intercalated long day.

A further experiment was designed to discover whether the inhibitory effect of a long day could be counteracted by following it immediately by a long dark period. For this purpose the treatment of the first experiment described was selected in which short days and long days were alternated until a total of 12 short days had been given. In the present experiment a 24-hour dark period was then interposed between these two cycles, (1) following the long day, and (2) preceding it. A straight alternation of long days and short days (3), and short days followed by a dark period of 24 hours (4), served as control treatments for (1) and (2).

The four cycles therefore were in hours light (L) and dark (D):

(1) 8L, 16D, 16L, 32D × 12
(2) 8L, 40D, 16L, 8D × 12
(3) 8L, 16D, 16L, 8D × 12
(4) 8L, 40D × 12

The results of this experiment are shown in Table VI.

TABLE VI

Effect of 24-hour Dark Periods preceding or following Long Days themselves alternating with Short Days. Twelve Replicates per Treatment

Treatment	Number of plants budded	Mean number of flowers per plant	Reciprocal of number of days to budding
(1)	12	158·1	0·026
(2)	5	2·5	0·011
(3)	2	0·75	0·0047
(4)	12	174·6	0·033

It will be seen from this table that a long dark period following the long day (treatment 1) very largely neutralizes the inhibitory effect of the unfavourable cycle and flowering is practically as intense as in treatment (4), which consists of a short photo-period and very long dark period. The fact that 24 hours' dark following the short day (2) (i.e. preceding the long day) hardly increased flowering at all compared with a straight alternation of long days and short days (treatment 3) shows that the effect is almost entirely due to the dark period destroying the inhibition produced by the long day—an effect it cannot produce when preceding it. In any case 16 hours' light followed by 32 hours' dark would not seem to constitute a cycle itself suitable for flower induction since otherwise treatment (1) would have had twice the number of inductive cycles as (4), which should have become apparent in the flower numbers. Schmitz (1951) also records this combination as vegetative.

In the last experiment to be described an induction period of 12 short days was split into two groups of 6 short days each by a varying number of long days, i.e. 0, 1, 2, 3, 4, 6, 8, 12, and 16. It was hoped that this experiment would indicate whether an unfavourable long day can do more than annul the effect of the $1\frac{1}{2}$–2 short days immediately preceding it (or following it), i.e. whether

the long days given together have the same effect as when each follows on and precedes a short-day induction period, though this was thought unlikely in view of the non-accumulation of the inhibitory effect. The data resulting from this experiment are recorded in Table VII. As this experiment was carried out during the winter, flower numbers were generally low even in the controls.

TABLE VII

Effect of varying Numbers of Long-day Cycles interposed between Two Groups of Six Short Days each. Ten Replicates per Treatment

Number of interposed long days	Number of plants budded	Mean number of flowers per plant	Mean number of days to budding
0	10	24·0	24·5
1	10	17·2*	25·3
2	10	13·4	26·0
3	10	17·6	24·8
4	10	14·8	28·6
6	10	14·8	26·8
8	10	12·7	25·7
12	10	11·5	26·0
16	10	8·8	24·3

* If one very aberrant plant is included this figure would amount to 21·4 flowers.

As was expected, the data shown in Table VII confirm that the effect of several long days given consecutively is not cumulative, the inhibitory effect of one long day being nearly, if not quite, as large as that of several. If, however, a large number of long days is intercalated, e.g. 12 or 16, flower numbers appear to be reduced further, resembling more those from less induced plants. In this connexion it should be noted that the average time required for the macroscopic appearance of the inflorescence was just over 25 days from the beginning of treatment, and presumably by the end of the interposed long-day period inflorescence initiation in these treatments must have been well advanced in consequence of the first 6-day induction period; it would seem possible therefore that flower numbers were already determined before the second set of 6 short days was received. In fact it would be interesting to discover to what extent partially induced plants can still benefit from further induction after delays of different duration in conditions unfavourable for flowering.

DISCUSSION

Before discussing the results of the *Kalanchoe* experiments described the main facts established may be listed:

(*a*) Single long days intercalated between numbers of short days have a positive inhibitory effect on flower initiation and are not merely ineffective.

(*b*) The inhibitory effect expressed as the number of inductive cycles

annulled is approximately additive, provided the long days are interspersed with short days, but not if several long days are given consecutively.

(*c*) The average inhibition due to 1 long day is capable of annulling the flower promoting effect of about $1\frac{1}{2}$–2 short days.

(*d*) To a first approximation flower numbers in *Kalanchoe* increase exponentially with the number of inductive cycles given—up to at least 12 short days.

(*e*) The inhibitory effect of long days interspersed with short days also fits an exponential curve; i.e. the inhibition is roughly proportional to the amount of previous photoperiodic induction.

(*f*) A light break of as little as 30 seconds' duration given in the middle of a long dark period is as inhibitory as a long day or continuous light.

(*g*) If followed by a long dark period the inhibition of an intercalated long day is almost completely neutralized; a long dark period preceding it has no such effect.

Nearly all theories on the mechanism of photoperiodic reactions are concerned with the effects of light and dark treatments during a single photoperiodic cycle (Gregory, 1948; Hamner, 1940; Harder and Bode, 1943; as well as Bünning, 1950), and it is only a stable end product or flower hormone which is considered to accumulate. The unfavourable effect of non-inductive cycles has been envisaged as due to the destruction during each cycle of some intermediate substance which in short-day plants is usually postulated as photolabile. The existence of inhibitory effects has of course been postulated previously on several occasions, e.g. Lang (1942) has suggested this for the long-day plant *Hyoscyamus niger* kept in short day, the annual variety of which can be made to flower under these conditions by defoliation. Recently Wareing (1954) has also interpreted some experiments on the effect of light breaks in long dark periods by suggesting that an inhibitor is formed. He and Carr (1951–2) would modify Gregory's scheme by postulating the interaction of two substances under unfavourable conditions. No clear suggestion is made, however, concerning the eventual fate of the inhibitor '*X*' which in soy-bean is said to be 'stable for a considerable period' and which therefore, one might surmise, should accumulate under prolonged unfavourable conditions (Wareing, 1954). The somewhat different behaviour of *Xanthium*, it is claimed, may be 'possibly due to the fact that (*it*) [this substance] is unstable or is utilized at a high rate and hence cannot exceed a certain maximum concentration even when the light period is prolonged'.

Similarly, auxin hypotheses of flowering have generally regarded growth substances as inhibitors of flowering (cf. the review by Bonner and Bandurski, 1952).

The present experiments supply positive evidence of the inhibitory effect of long days extending—at least as far as *Kalanchoe* is concerned—well outside

the cycle in which such conditions are given, affecting the product either of preceding or of succeeding inductive cycles. Also a quantitative estimation of the inhibition has been attempted. The fact that the amount of inhibition due to a single long day or interrupted dark period is quantitatively limited also removes one of the objections that have been raised in the past against photoperiodic theories involving inhibitors. For, such a limitation explains the non-occurrence of any so-called 'counter-induction' (e.g. Borthwick, 1947), the absence of which tended to argue against positive inhibition. In *Kalanchoe* one unfavourable cycle annuls about $1\frac{1}{2}$–2 favourable cycles; this appears to be so regardless whether it consists of continuous light, a long day, or a light break of 30 seconds. The inhibition is not additive and no accumulation takes place if a number of long days succeed one another, but if interposed between short days each will 'destroy' the effect of approx. $1\frac{1}{2}$–2 short days, so that an alternation of short days and long days will prevent flowering almost completely.

The possibility of summation of the inhibitory effect in this manner is particularly interesting in view of the exponential increase in flower numbers with increasing induction. Thus the amount of inhibition appears to be proportional to the degree of previous induction. In the first experiment the regression coefficient of the log. of flower numbers on the days of induction was 0·323 (antilog. = 2·1), which indicates on the average a doubling of flower numbers for each inductive cycle.

In the second experiment the coefficient was slightly lower (0·186; antilog. = 1·53), which is probably due to complete failure of all plants to flower in this experiment when only two inductive cycles were given.

The regressions for the effect of each long day in the first experiment had a coefficient of 0·331 (antilog. = 2·14), in the second experiment 0·447 (antilog. = 2·8). Hence flower numbers were reduced by factors of 0·47 and 0·36 by each interposed long day. It might perhaps be argued that the approximate doubling of flower numbers for each inductive cycle may be related to the morphology of the inflorescence of *Kalanchoe* (dichasia terminating in cincinni) and that each short day would in fact cause an arithmetic increase in the degree of branching, though this would hardly serve as an explanation in terms of a substance quantitatively determining flowering at an exponentially increasing number of sites. The observation that a 24-hour dark period following a long day (i.e. 32 hours' continuous dark) is almost completely effective in neutralizing the inhibitory effect suggests that the inhibition does not affect the product of the preceding cycle at all, but rather that of the next one in succession. Also the effect of the 24 hours' dark period cannot be due to this treatment merely representing an additional favourable cycle, because in that case it should be equally effective when it precedes the intercalated long day. However, in this connexion one other observation must be taken into account, i.e. it was found by Schmitz (1951) and the author (Schwabe, 1955) that a very long light period (20 hours) did not lead to flowering in *Kalanchoe* even if followed by as much as 30 hours' dark, from which it was concluded that

there was an upper critical light period at about this level as well as a critical dark period. Possibly, therefore, in treatment (3), page 2, the two main light periods and the intervening short dark period might have added up to an effective long light period exceeding the critical length, which in treatment (1), 16 hours' light followed by 32 hours' dark, did not do so. However, this is not very likely to be the case, since Schmitz failed to obtain any flowering with this combination, and in the present experiments also 24 induction periods would have been received by the plants in this treatment (if this combination had constituted an inductive cycle) and greater flower numbers would have been expected than in the controls with 12 inductive cycles consisting of 8 hours' light and 40 hours' dark. It seems very probable therefore that the inhibition affects only succeeding inductive cycles and not the product of preceding ones.

Any other explanation (e.g. temporarily reversible inactivation of the product of one or more preceding favourable short days) would be very difficult in view of the stability of the product in the main light period of continuous short-day treatment, and particularly in view of the quantitative limitation of inhibition.

Recently Gregory, Spear, and Thimann (1954) have reported an entirely new effect of short-day treatment on the metabolism of *Kalanchoe*. They have demonstrated that CO_2-fixation in the dark is quantitatively dependent on short-day treatment and is almost entirely suppressed by long days or by short days with interrupted nights. In a yet tentative explanation they postulate the formation of a CO_2-acceptor in the light period and gradual evolution of an enzyme catalysing the fixation reaction. The enzyme system, it is suggested, builds up gradually under the influence of increasing numbers of inductive cycles, but long-day treatment or light breaks prevent its formation.

On the basis of some of the results and theories mentioned above the following eclectic scheme might be proposed which would at least fit the facts revealed by the present experiments. To devise such a scheme would not appear to be entirely unprofitable, though the writer would be much surprised if many weeks should pass before new facts either prove its complete failure or demand drastic modifications.

The following assumptions are made:

(*a*) A reaction catalysed by an enzyme E and yielding a flower promoting product might be imagined as follows: $CO_2 + Y \rightarrow Z$. This reaction might be thought of as similar to that of Gregory et al. (loc. cit.). It proceeds only in the dark, Y being a product of reactions requiring high light intensity, being possibly identical with pyruvic acid.

(*b*) The formation of enzyme E is adaptive and increasing amounts of it are formed with increasing activity.

(*c*) A second reaction may be imagined leading to the formation of an inhibitor I. This reaction may generally be similar to that suggested by Wareing and Carr (1951–2); it would be photochemical, but require only low light intensities. It would depend on a precursor formed

independently of light or darkness, but an upper limit to the possible amount of precursor must be assumed. In addition *I* would need to be gradually destroyed, or translocated away from the leaves in the dark.

(*d*) The *I* formed in the light combines competitively with enzyme *E*, thus blocking reaction (*a*). In the dark *EI* dissociates as the level of *I* falls, enabling *E* to catalyse reaction (*a*).

In continuous short days then *I* would generally remain at a fairly low level; thus *E* would catalyse the formation of *Z* and itself increase exponentially to a high level, flower promotion increasing at the same rate. Also, after an adequate induction period so much *E* would be present that even in unfavourable conditions not all of it could be inhibited.

In long days or short days with interrupted nights an excess of *I* is present at all times and what little *E* may be present remains blocked in the form *EI; E* itself does not increase in amount.

When a run of short days is interrupted by intercalated long days, *E* is blocked and *I* reaches the maximum level in the interrupting cycle. In this case a very long dark period, i.e. of the order of 30 hours or more, is required to dissipate *I* again and for *EI* to dissociate. Hence an ordinary short day following on a long day would remain ineffective in view of the excess of *I*, and if the estimate that 1 long day $\sim$ to $1\frac{1}{2}$–2 short days is valid, even in the dark period of the second short day *E* would not operate at full efficiency. Also the multiplication of *E* would be prevented until at least the second short day following the interposed long day, i.e. *E* would be retained at the level present. This would account for the proportionality between the inhibitory effect and the degree of previous induction.

With very long light periods followed by very long dark periods failure to flower would initially be due to *I* being at its maximum. If the dark period following is long enough to allow for removal of all *I*, *Y* itself may have been dissipated or translocated away, as is postulated for the CO_2 acceptor by Gregory et al.

This scheme then could account for the effect of long days annulling short days following them, the effect being proportional to the previous induction.

No doubt other schemes could be devised to fit the same facts and the one presented is not intended to do more than indicate a possible example.

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Studies in the Physiology of Parasitism

XX. The Pathogenicity of *Botrytis cinerea*, *Sclerotinia fructigena*, and *Sclerotinia laxa*, with special reference to the part played by pectolytic enzymes

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With six Figures in the Text

ABSTRACT

1. Though *Sclerotinia fructigena*, *S. laxa*, and *Botrytis cinerea* cause rotting of apple tissue and death of the protoplasts, little or no pectolytic activity was detectable in extracts of the rotted tissue.

2. Pectic materials were extracted from normal and parasitized apple tissue in three fractions and precipitated as calcium pectate. There was a loss of total, total insoluble, and soluble pectic substances in the invaded tissues. This was most marked with *B. cinerea* and *S. laxa* and least with *S. fructigena*.

3. Pectolytic activity was measured by methods involving (*a*) maceration of plant tissues, (*b*) viscosity and reducing group determinations in pectic substrates, (*c*) increase in acidity of pectin. By these methods it was shown that pectolytic enzymes were produced by all three fungi in synthetic media. With *S. fructigena*, which was the only fungus studied in detail, replacement of glucose by pectin increased the formation of pectolytic enzymes.

4. When various apple extracts were used as culture media, little or no pectolytic activity was detectable. With all three fungi the presence of apple juice in a culture medium, which by itself was suitable for enzyme formation, resulted in the suppression of pectolytic activity.

5. Oxidized apple juice had a pronounced effect in deactivating certain pectolytic enzymes, an effect which was especially marked with *B. cinerea*. This points to an interaction between the pectolytic and oxidizing systems and introduces a new line of approach to the study of the biochemical interaction between host and parasite.

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Introduction

THE 'Brown Rot' diseases of the common prunaceous and pomaceous fruits are characterized by a perceptible rotting of the tissue in the earlier stages of attack, but later by a process of hardening which leads finally to the well-known 'mummified' fruit. The parasites causing these diseases therefore stand in distinct contrast to organisms such as *Botrytis cinerea* and *Bacterium carotovorum*, which cause a more or less pronounced soft rotting of the host tissue invaded. From the latter, or from cultures of the parasites concerned, active solutions of pectolytic enzyme can be prepared with ease; on the other hand, attempts to prepare similar extracts from the 'Brown Rot' fungi or from fruit rotted by them have led to uncertain results. In view of this contrast, it appeared desirable to make a fresh study of the pectolytic enzymology of the 'Brown Rot' fungi, especially as recent advances in pectin chemistry had furnished a range of fairly well-defined substances which could serve for the characterization and estimation of these enzyme systems. A summary of the present position of pectin chemistry and of the pectolytic enzymes[1] which have been characterized has been given by Wood (1955) in an earlier number of this series.

Valleau (1915), on the basis of photomicrographs of rotted plums, concluded that *Sclerotinia cinerea* (= *S. laxa*) followed the line of the middle lamella when penetrating tissue. He stated that the host cells became separated from each other ahead of the hyphae by something secreted by the fungus, but he failed to demonstrate the presence of a macerating enzyme in extracts of the rotted tissue.

Muhleman (1925) showed that aqueous extracts of the mycelium of *S. cinerea* contained a macerating enzyme, but he could not detect it in the prune-juice culture medium.

Davison and Willaman (1927) used many cultures of *S. fructigena* and *S. cinerea* but failed to detect any macerating principle either in the ground mycelium or in culture media, which in most cases were fruit decoctions such as prune juice. They did, however, demonstrate the presence of a pectolytic enzyme in the culture medium which increased the reducing power of a pectin solution in 24 hours. Therefore PG was presumably present; they called it pectinase.

Menon (1934) grew *S. fructigena* and *Botrytis cinerea* on autoclaved apple plugs and obtained macerating enzymes from the juice of the tissue rotted by each of the fungi.

Vasudeva (1930) grew *Botrytis allii* on apple-extract medium either as such or with addition of asparagine or potassium nitrate at 0·4 per cent. concentration. He found that the maceration activity of the culture filtrates when tested on potato or apple discs was very low, even with nitrogen in the medium. When,

[1] The enzymes Pectinesterase, Polygalacturonase, and Depolymerase will be referred to as PE, PG, and DP respectively.

however, he used aqueous extracts of the dried mycelium he found that although mycelia from cultures on apple extract alone had little macerating activity, those from cultures to which nitrogen was added contained appreciable amounts of the macerating enzyme. He also showed that, on inoculating apples with *S. fructigena* and *B. allii*, the addition of nitrogen to the inoculum increased the subsequent rate of attack by the former and led to an attack by the latter, a fungus which does not normally invade apples. He concluded that the addition of nitrogen to the apple tissue and apple extracts, which normally contain little nitrogen, resulted in increased formation of pectolytic enzymes.

Bray (1938) grew *B. cinerea* on plugs of living and steamed apple tissue and from the results obtained by testing the juice from the rotted tissue with potato discs concluded that very little macerating enzyme was produced by *B. cinerea* when grown on apple tissue.

The main body of the evidence from the literature cited shows that when the fungi used in this investigation are grown either on certain fruit tissues (prune, apple) or on decoctions of these, little macerating enzyme is demonstrable either in the juice from the rotted tissue or in the culture media. The only case in which apparently measureable quantities have been reported from *S. fructigena* and *B. cinerea* grown on apple tissue is that of Menon (1934). Unfortunately it is not possible to determine from his results the actual maceration times of the extracts used. However, there is some evidence that pectolytic enzymes are formed, as they have been reported from aqueous extracts of the dried mycelium of all the fungi.

There are many reports of *B. cinerea* secreting pectolytic enzymes into culture media other than those of fruit origin, e.g. when grown in turnip juice (Brown, 1915), in potato decoction (Bray, 1938), and in artificial media (Fernando and Stevenson, 1952; Tribe, 1955). As regards *S. laxa* there does not appear to be any other work in this connexion beyond the papers cited above.

In recent years considerable attention has been paid to the question whether particular fungi produce certain enzymes 'constitutively', i.e. independently of the presence in the medium of the substrate of the enzyme, or 'adaptively', i.e. the enzyme being produced only when its particular substrate is present. Some of the work has related to pectolytic enzymes; the following papers can be cited in this connexion.

Harter and Weimer (1923) found that the substitution of pectin for glucose in Czapek medium resulted in the production of macerating enzymes, but none was detected when glucose alone was used. With pectin and glucose together, enzyme production was feeble.

Proskuriakov and Ossipov (1939) used ground dried mycelium of *B. cinerea* and *S. fructigena* as sources of pectolytic enzymes, and demonstrated that the presence of pectin in the medium increased their formation. They further concluded that the PG and PE enzymes were at least partially adaptive.

Gäumann and Böhni (1947) reported that in cultures of *B. cinerea* pectinase

(PG) was produced constitutively. Pectase (PE), however, was formed adaptively.

Phaff (1947) claimed that both PG and PE were produced adaptively by *Penicillium chrysogenum* but not in the strictest sense, inasmuch as their formation was conditioned by a number of substances such as pectin, pectic acid, D-galacturonic acid, mucic acid, &c., all of which, however, are closely related to pectin.

These reports indicate that both *B. cinerea* and *S. fructigena* produce pectolytic enzymes in suitable culture media and that the presence of pectin in the medium may increase the yield. It is all the more remarkable that little of these enzymes has been reported from cultures on apple media, as these would certainly contain reasonable amounts of pectin.

Materials and Methods

Of the three fungi used, *Sclerotinia fructigena* and *Botrytis cinerea* were isolated from apple fruit by the writer at the beginning of this work; the culture of *S. laxa* was obtained from East Malling Research Station and was originally isolated from nectarine. The purity of all three was ensured by making single spore or hyphal tip cultures.

Stock cultures were set up on potato extract agar, allowed to grow at 25° C. for about a week, then stored at — 20° C. in a refrigerator. So treated they remained viable for at least 6 months.

In order to obtain spores in large quantities, somewhat different methods had to be adopted for the three fungi. For *B. cinerea*, best results were obtained by spreading a suspension of spores over the surface of potato extract agar slopes in boiling tubes. These were then incubated at 20° C. for 5 or 6 days. For *S. fructigena* and *S. laxa*, potato extract agar acidified with 1 per cent. malic acid was used. Slopes were inoculated with a single inoculum in the centre, as the method so effective for *B. cinerea* yielded few or no spores from these fungi. It was also necessary to incubate in daylight. In 10 days, 7 or 8 concentric rings of spores were formed.

Pathogenicity tests were almost entirely carried out with 'Bramley Seedling' apples, which were obtained from cold store at East Malling Research Station and were available from October to March in each year. To a much less extent, parallel tests were made with 'Conference' pears and unripe 'Victoria' plums.

Fruits were inoculated after the method described by Granger and Horne (1924). The depth of the inoculum was standardized by the insertion of a metal rod to a set distance inside a cork borer of 0·6 cm. diameter. A plug of tissue was removed and in its place was inserted a plug of agar cut from the outer edge of a 10-day-old Petri-dish culture on potato extract agar. The wound was sealed with a warm vaseline-wax mixture.

Extracts of parasitized tissue were prepared by scraping out the rotted part at a time when the parasite was still actively progressing. The juice was

squeezed through muslin and centrifuged at 10,000 r.p.m. for 5 minutes. Extracts from non-parasitized fruit were made by mincing and then clearing as just described. Specially prepared extracts from fresh fruit were also made in another connexion, as will be described later (p. 33).

Cultures on liquid media were also assayed for content of pectolytic enzymes. The medium was placed in medicine flats, 30 ml. to each, which after inoculation were incubated on their sides. With *B. cinerea* and *S. laxa* the inoculum consisted of a dense suspension of spores, as these were available in quantity at all times of the year. With *S. fructigena* a similar method was adopted at first, but it was more troublesome to obtain spores of this fungus in quantity, especially during the dark months. An inoculum of spores was replaced by ten agar plugs, cut with a cork borer of 0·5 cm. diameter, from the outer edge of a 10-day-old Petri-dish culture on 0·5 per cent. glucose agar. Preliminary experiments showed that this method was as satisfactory as that using spore inocula, except that a slightly longer incubation period was necessary. At the end of the incubation period the enzymic activity of the filtrates was determined at once, or they were stored at — 20° C. until required. The mycelial mats were thoroughly washed, dried at 70° C., and weighed.

Enzyme activity was measured both on plant tissues and on soluble pectic substances. For estimation of maceration activity the method used was that described by Brown (1915). Prepared tissue discs[1] (apple, cucumber, potato, turnip) were immersed in the test solution, which was buffered at the required pH with McIlvaine's phosphate-citrate buffer. The maceration time in minutes (average of six discs) was taken as that required for the discs to lose coherence under a slight pull. Activities were recorded as reciprocals of these times × 100.

For the determination of toxic effect on tissue cells a technique described by Tribe (1955) was used. The tissues were immersed in a plasmolysing solution containing 9 ml. of 0·5 M. sucrose, 1 ml. of 0·1 per cent. neutral red, and buffered at pH 8·0 with McIlvaine's buffer. Cells were considered to be dead if the protoplasts did not plasmolyse or take up the stain.

Fou rsoluble pectic substances were used, of which three were commercial preparations, as follows:

Lemon pectin—from General Foods Corporation; 12·3 per cent. methoxyl.

Apple pectin—from H. W. Carter & Co.; 9·7 per cent. methoxyl.

Sodium ammonium pectate, known as 'sodium polypectate'—from California Fruit Growers Exchange; 1·3 per cent. ammonium, 4·2 per cent. sodium.

[1] Although apple discs were the obvious choice of tissue for maceration tests in this work, they could only be used when unripe owing to the liability of ripe tissue to disintegrate in water. This feature severely limited their availability for the purpose on hand. Preliminary tests showed that maceration which occurred when unripe apple discs were immersed in an active enzyme solution was comparable to that which occurred in potato and other tissues.

In order to ensure their purity, these substances were washed with 80 per cent. ethyl alcohol acidified with hydrochloric acid, as described by Kertesz (1951, p. 113).

The fourth substance was a sodium pectate prepared in the laboratory by a method quoted by Kertesz (1951, p. 122), and originally given by Lineweaver and McCready (1945). A 3 per cent. solution of Carter's apple pectin was maintained at pH 10·0 with 0·5 N. sodium hydroxide solution until the pH did not tend to alter appreciably. The solution, so de-esterified, was stored under toluene and used as such, i.e. without precipitation to powder form and redissolving. Its viscosity was the same as that of a 1·6 per cent. solution of apple pectin, the loss of viscosity being due to some degradation of the pectin by de-esterification with alkali.

Viscosity determinations, for assessment of PG or DP enzymes, were made with an Ostwald viscometer at 25° C. in a water-bath. The technique has been outlined by Wood (1955). PE enzyme was determined by titrating liberated carboxyl groups with alkali, after the method of Kertesz (1937).

pH values were determined either colorimetrically with standard B.D.H. indicators or with a pH meter.

Effects of Parasites on Host Tissues

Apples, pears, and plums are all attacked by each of the three fungi used, with the production of rotted brown tissue. This was examined for the presence of pectolytic enzymes and was also analysed for its content of pectic

Table I

Rate of Invasion (g.) of Apple Tissue

	5° C.	15° C.	20° C.	25° C.
S. fructigena	nil	33	83	126
S. laxa	nil	26	46	61
B. cinerea	1·6	29	25	18

substances, as compared with normal tissue. The results are set out in this section.

Table I gives the amounts of rotted tissue, scraped out and weighed, of 'Bramley Seedling' apples inoculated in February with each of the three fungi. The data are the average weights of rotted tissue after 8 days' incubation at the temperatures shown. The experiment was run in sextuplicate.

The above figures show that *S. fructigena* is the most active parasite, *B. cinerea* the least, while *S. laxa* occupies an intermediate position. The difference is most marked at the highest temperatures; at lower ones the rates of attack become approximately the same, until at 5° C., a cold-store temperature, *B. cinerea* alone attacks. With comparatively unripe apples, e.g. with 'Bramleys' in September or October, these differences in rate of invasion of apple fruit by the three fungi are more marked still, *B. cinerea* invading such

apples only very slowly. On the other hand, early varieties such as 'Devonshire Quarrenden' are readily attacked by *B. cinerea* in July and August. These results are in agreement with general experience that *B. cinerea* has negligible significance as an apple parasite until late in the season, and especially in cold store, whereas the two Sclerotinias, and *S. fructigena* in particular, may be rampant on unripe fruit throughout most of the growing season.

Results of inoculations of pears and plums showed the same general trends but were carried out on quite a small scale.

The rotted tissue was brown, both at the surface and inside. The only exception to this was with apples invaded by *B. cinerea* at 5° C. At this temperature the invaded tissues were only slightly brown, this presumably being the result of slow oxidase action at the low temperature. Each of the three fungi, when they invaded apple tissue, produced a rot which was fairly characteristic. Radial sections through the point of inoculation revealed the outline of the advancing mycelium with the rotted tissues behind. With *S. fructigena* and *S. laxa* the outline tended to be irregular, with fungal growth most rapid in the regions of the vascular bundles; with *B. cinerea* the outline was more regular. Tissue invaded by *S. fructigena* was only slightly less firm than normal tissue; *B. cinerea* and *S. laxa* produced a soft rot, which could easily be scraped away from uninvaded tissue.

In order to determine the effect of the advancing mycelium on cells of the host tissues, thin hand sections were cut along the line of fungal advance, in the region of invading hyphae, and plasmolysed in 0·5 M. sucrose. Intercellular air was removed under the air pump and the sections placed in the sucrose and neutral red preparation described above (p. 19). The appearances presented, with all three fungi, and on all three fruit tissues, were essentially the same. Fig. 1 shows the effect of *S. laxa* on apple tissue. The fungal hyphae are advancing from the left-hand side towards the right. The living protoplasts, which are plasmolysed and stained, can be seen as distinct dark circles. There is therefore evidence for the presence of a toxin in the region of the advancing hyphae and behind them. Further evidence of the death of invaded tissues is the browning, which is the result of post-mortem oxidation.

For enzymic studies, apples were inoculated and incubated at 25° C. for periods which varied for the fungus concerned, viz. 4, 5, and 7 days for *S. fructigena*, *S. laxa*, and *B. cinerea* respectively. By this means approximately equal amounts of rotted tissue were given by the three fungi. Extracts of rotted tissue were prepared, as already described, and tested for pectolytic activity by their action on apple and potato discs and by the viscosity method with sodium polypectate. Both these activities were determined at pH 6·0. For PE tests, the extracts were precipitated in acetone at 5° C., the precipitate centrifuged off, dried in air, and redissolved in a volume of water equal to the original volume of the extract. The data are given in Table II.

Maceration activity was slight for *B. cinerea* and not definitely proved for

the *Sclerotinia* spp. PG activity was shown by a small drop in viscosity after 1 hour. PE activity was so slight that the reaction was continued for 20 hours instead of the usual 1 hour.

By way of contrast to the results of Table II a sample set of data referring to enzyme preparations from culture solutions (see p. 24) are shown in Table III.

In all respects, therefore, the extracts of rotted apple tissue, as shown in Table II, are extremely weak in pectolytic enzymes. Similar measurements were made with apple tissues rotted at different temperatures (from 5 to

FIG. 1. Effect of *S. laxa* on apple tissue. For explanation see text.

25° C.) and at different stages of ripeness of 'Bramley' fruit (Oct.–March). In some of these the extracts were prepared both from tissue near the point of inoculation and from the region of the advancing hyphae, but none of the extracts so obtained was more active than those shown in Table II. Some of them, including those from *B. cinerea*, were in fact completely inactive with respect to maceration and viscosity tests.

The percentage of pectic materials present in normal and parasitized apple tissue was determined in the following way. Batches of 20 apples were inoculated with each of the three fungi and 20 kept as controls. Incubation was at 20° C. for 12 days. The rotted tissues were scraped out, minced twice to ensure adequate mixing, then stored at −20° C. The controls were treated similarly.

The method used for the extraction of pectin from the parasitized apple tissue was that described by Carré (1925). Modifications of the original

TABLE II

Pectolytic Activity of Extracts from Parasitized Apple

	Maceration	Viscosity		PE
		Initial	Final	
S. fructigena	<0·1	62	60	0·05 ml.
S. laxa	<0·1	62	54	nil
B. cinerea	0·1	62	43	0·2 ml.

Carré and Haynes (1922) method, for the precipitation of pectin as calcium pectate, suggested by Kertesz (1951), were incorporated. The pectic substances were extracted in three fractions, viz.

(i) Those soluble in cold water and capable of being washed from the tissue.
(ii) Those soluble in water after restricted hydrolysis with dilute acid.
(iii) Those soluble in water after restricted hydrolysis with alkali.

TABLE III

Pectolytic Activity of Culture Solutions

	Maceration	V. 75*	PE†
S. fructigena	3·3	36	1·2
S. laxa	0·3	1·5	1·0
B. cinerea	6·7	200	2·0

* Inverse of time (minutes) required to reduce initial viscosity of pectic solution by 75 per cent., this figure then multiplied by 100. A V. 75 figure of 36 means that 75 per cent. of viscosity was lost in less than 3 (100/36) minutes. The preparation of *B. cinerea* achieved the same effect in half a minute.

† Measure of acidity produced in 1 hour as against the 20 hours of Table II.

Carré (1925) describes these fractions as containing, respectively, (i) the original soluble pectin of the apple, (ii) the original insoluble pectic material of the primary cell walls, which she termed 'pectose', (iii) the original pectic material comprising the middle lamella between adjacent cells.

The accuracy of Carré's method was tested by adding a known weight of apple pectin to water or to extracts of apple tissue. A recovery of 93–105 per cent. was obtained by the method.

Table IV gives the average weights (from duplicate precipitations) of calcium pectate obtained from 10 g. (dry wt.) samples of apple tissue. Subsidiary tests showed that within the time limits of the experiment there was no appreciable change in percentage dry matter as a result of parasitism by any of the fungi. The figures of Table IV are therefore comparable in terms of fresh weight of tissue.

The figures in the last and last but one columns of Table IV show that there is a loss of total and total insoluble pectin, in the tissue invaded by all three fungi, compared with that of the control. This loss is much more marked in tissue invaded by *B. cinerea* and *S. laxa* than by *S. fructigena*, viz. a loss of 16·5 per cent. of the total insoluble pectin for *S. fructigena*, 68 per cent. for *S. laxa*, and 66 per cent. for *B. cinerea*. The figures thus substantiate the observation on the relative firmness of the invaded tissues noted above.

TABLE IV

Pectic Content (mg). *of Apple Tissues*

	Soluble pectin	Acid treated	Alkali treated	Total insoluble	Total
S. fructigena . . .	29	268	279	547	576
S. laxa	61	154	57	211	272
B. cinerea . . .	19	113	111	224	243
Control . . .	183	609	46	655	838

The soluble pectin has been decreased in all the invaded tissues, and the acid-treated portions show similar results. It is difficult to explain the large amounts of pectin extracted by alkali hydrolysis, in particular from tissue parasitized by *S. fructigena*, especially as Carré describes the pectin extracted by this method as middle lamella pectin. However, as degradation of the insoluble pectic materials primarily would affect the firmness of the parasitized fruit, the acid and alkali extracted portions may be considered together as insoluble pectin.

To sum up the position, it is clear that pectic materials of apple tissue are broken down by all the fungi used, that there is a partial loss of coherence of the tissue, and that the cells are killed. Little or no evidence, however, could be detected for the presence of pectolytic enzymes, capable of causing such effects, in the extracts of rotted tissues.

PRODUCTION OF PECTOLYTIC ENZYMES IN CULTURE MEDIA

Though little pectolytic activity was detected in apple tissue parasitized by the three fungi used, they all produce these enzymes when grown in suitable culture media. As chief interest centred on the mode of attack of *S. fructigena*, most of the cultural work was done with this fungus. The media used were (*a*) synthetic, with or without addition of pectin, and (*b*) apple extracts variously modified. They will be dealt with in that order.

The following information was recorded in all the experiments in this section: dry weight of mycelium in milligrams; maceration activity of culture media on potato discs, 0·25 mm. thick, at pH 6·0; viscosity activity (V. 50) of the same, with sodium polypectate as the substrate, also at pH 6·0; PE activity of the same on lemon pectin. Cultures were incubated for 11 days at 25° C. unless otherwise stated.

(a) Synthetic media

The results in Fig. 2 were obtained by growing *S. fructigena* in a basal medium consisting of 1·0 per cent. ammonium tartrate; 0·1 per cent. KH_2PO_4; 0·5 per cent. $MgSO_4.7H_2O$, and with glucose concentrations ranging from 0·1 to 2 per cent.

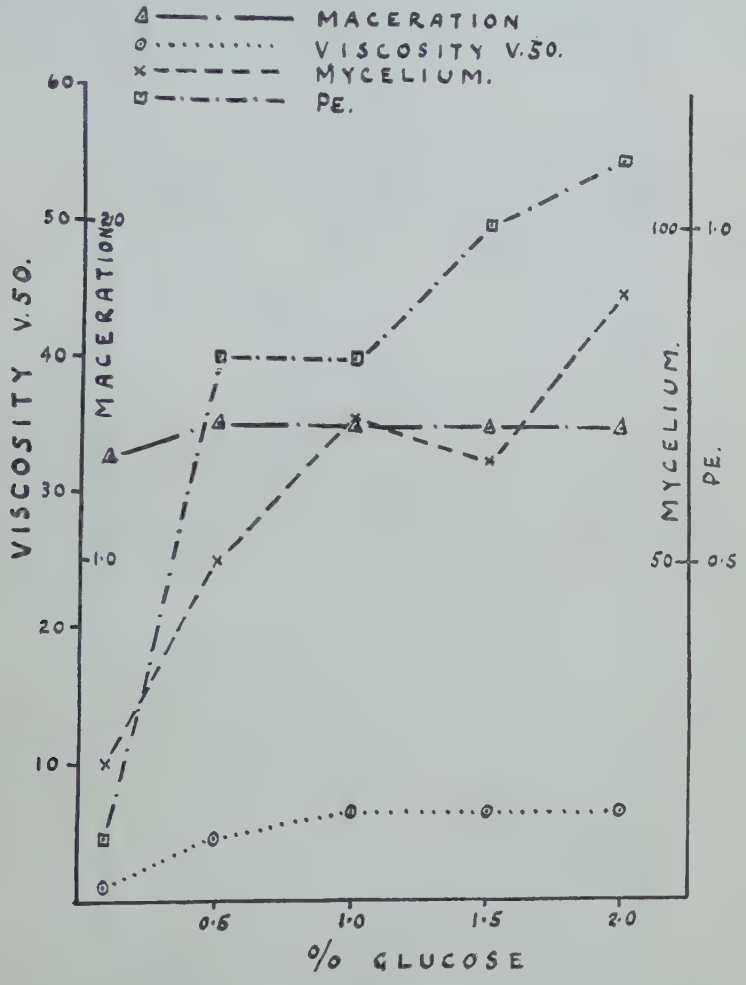

FIG. 2. Growth and pectolytic activity of *S. fructigena* in culture media containing various concentrations of glucose.

Growth and PE activity show a steady increase as the glucose concentration goes up, but maceration and viscosity activities remain constant at a fairly low level apart from a slight falling off at low concentrations of glucose. Thus there is a tendency for the maceration and viscosity activities to show parallel trends. The similarity of the trends in growth and PE activity is also noteworthy.

The initial pH of the media was 6·0. Growth of the fungus caused a drift towards greater acidity, viz. to 5·6 and to 4·6 for the media with least and greatest glucose concentrations respectively, with intermediate figures for the intermediate concentrations.

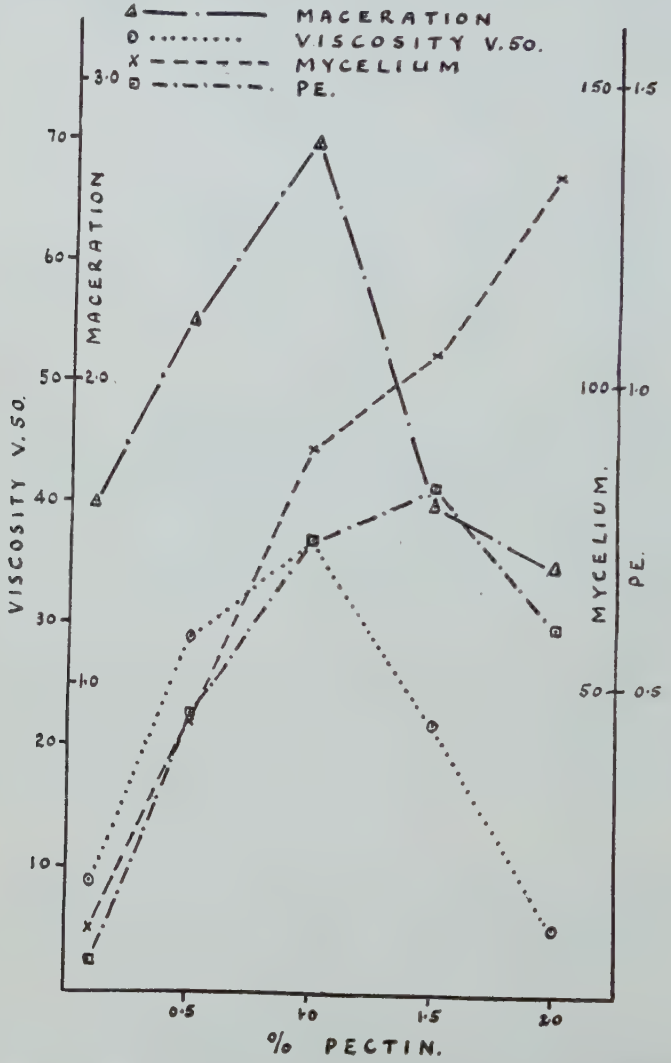

Fig. 3. Growth and pectolytic activity of *S. fructigena* on culture media containing various concentrations of apple pectin.

The effect of substituting apple pectin for glucose in the medium was also examined. This was studied in some detail, with reference to the effect of varied concentration of apple pectin, of different periods of incubation, and of different concentrations of ammonium tartrate and other nitrogen sources.

Fig. 3 gives the behaviour on media containing the following: 0·5 per cent.

ammonium tartrate; 0·1 per cent. KH_2PO_4; 0·05 per cent. $MgSO_4.7H_2O$, and apple pectin ranging from 0·1 to 2 per cent.

Mycelial weight continued to increase with increasing pectin concentration in the medium. Maceration and viscosity activities showed the same parallel behaviour as seen in Fig. 2, increasing up to the 1 per cent. pectin level, then falling off at higher concentrations. PE activities showed a similar trend to maceration and viscosity activities in this case, except that the maximum was at a slightly higher pectin concentration.

TABLE V

pH *Changes in Culture Media containing Pectin*

% Apple pectin	Initial pH	Final pH
0·1	5·4	6·0
0·5	4·8	5·8
1·0	4·6	5·8
1·5	4·4	4·8
2·0	4·4	4·4

It will be noted that maceration and viscosity activities shown at the peaks in Fig. 3 were considerably higher than those of Fig. 2, and this in spite of the lower concentration of ammonium tartrate in the media of Fig. 3 (see later in this section). Secretion of the macerating and viscosity-destroying enzymes (or enzyme) is thus stimulated by the presence of a suitable concentration of pectin in the medium.

As apple pectin is acidic, the initial pH of the culture solution falls with increasing pectin concentration (Table V). In contrast to the cultures containing glucose, the pH of those with pectin tended to increase towards the neutral point, most noticeably in the media containing 0·5 per cent. and 1·0 per cent. pectin. It is possible that the low pH at the higher concentrations of pectin was responsible for the reduced secretion of pectic enzymes causing maceration and loss of viscosity, as compared with the higher concentrations of glucose (Fig. 2).

Curves were also prepared showing the effect of incubation period—at daily intervals from the 7th to the 14th day—at three levels (0·5, 1, 1·5 per cent.) of pectin concentration. The outstanding result was the general parallelism throughout of the curves for macerating and viscosity-reducing activity. A high level of these activities was already shown by the 7th day in the medium with lowest pectin; by the 10th day the medium with 1 per cent. pectin had taken the lead, and by the 14th day the medium with 1·5 per cent. pectin, which had lagged throughout, had almost caught up with the others. Thus, within the limits examined, high concentration of pectin, though it increases mycelial weight, slows down the rate at which the macerating and viscosity-reducing enzymes are produced.

Fig. 4 records the data obtained by growing the fungus on media of basal composition: 1 per cent. ammonium tartrate; 0·1 per cent. KH_2PO_4; 0·05

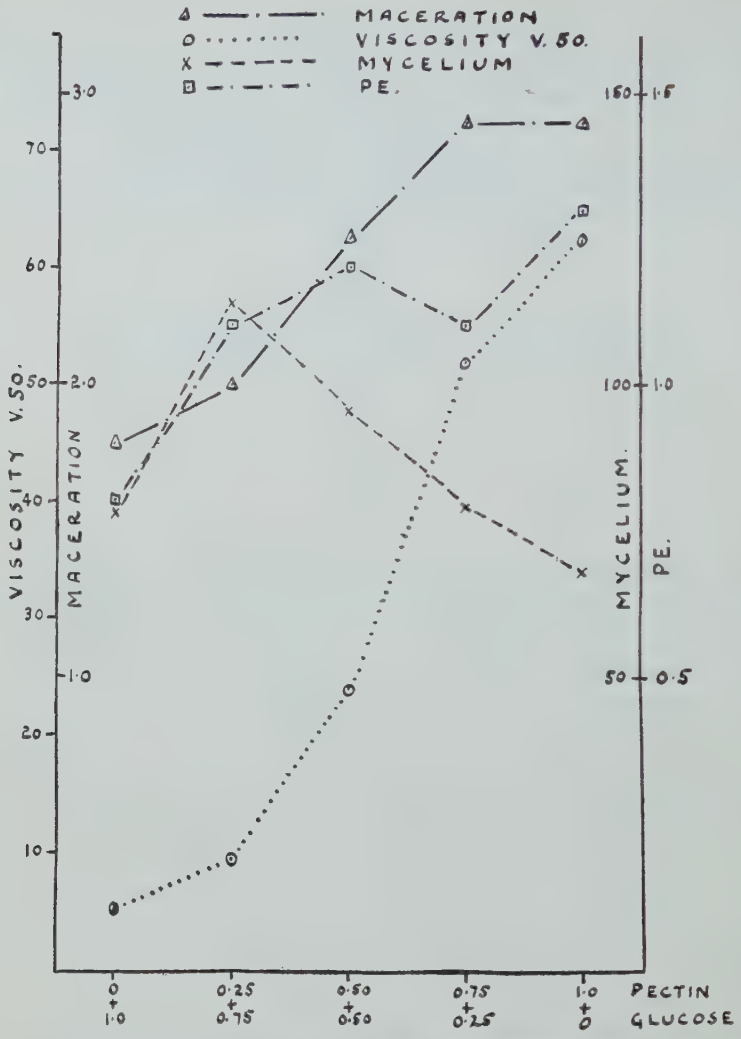

FIG. 4. Effect of glucose-pectin mixtures on growth and pectolytic activity of *S. fructigena*.

per cent. $MgSO_4.7H_2O$, together with a range of glucose-pectin mixtures, viz.:

Pectin %	Glucose %
1·0	0
0·75	0·25
0·50	0·50
0·25	0·75
0	1·0

In the media containing 1 per cent. pectin and 1 per cent. glucose alone, growth is about equal; it is maximal with 0·25 per cent. pectin+0·75 per cent. glucose. Pectolytic activity is greatest with pectin alone, and shows a steady

decrease as the pectin is replaced by glucose. This effect is even more marked if the enzymic activities are expressed per unit dry weight of mycelium.

In Fig. 5 the results refer to culture media containing the following: 1 per

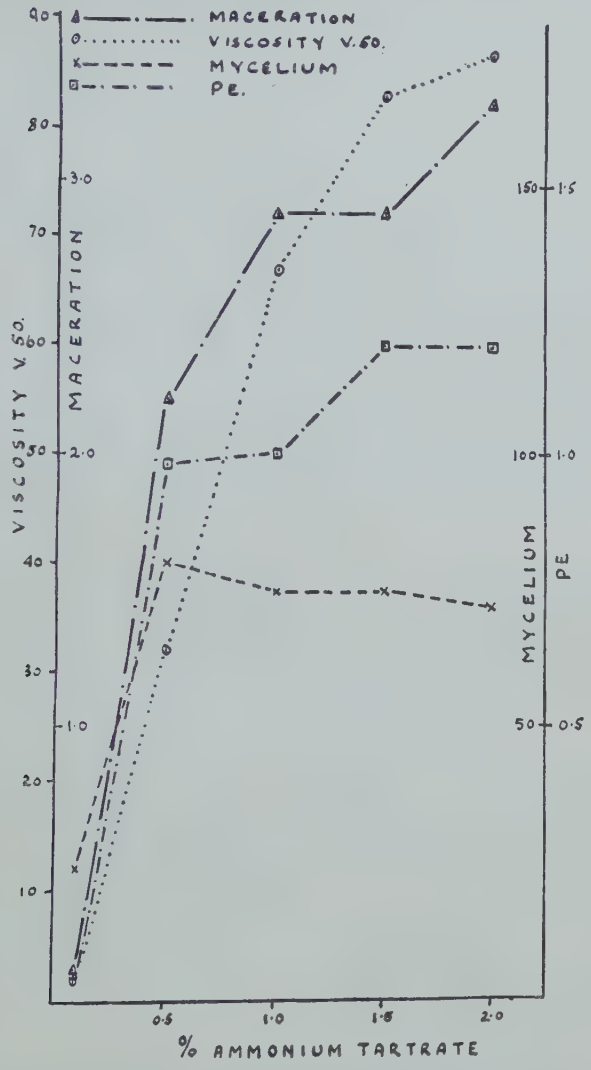

FIG. 5. Growth and pectolytic activity of *S. fructigena* in culture media containing various concentrations of ammonium tartrate.

cent. apple pectin; 0·1 per cent. KH_2PO_4; 0·5 per cent. $MgSO_4.7H_2O$; and concentrations of ammonium tartrate ranging from 0·1 to 2 per cent.

Once again it is seen that the macerating and viscosity activities follow the same general paths. There is little growth or pectolytic activity in the medium with 0·1 per cent. ammonium tartrate, but the maceration, viscosity, and to some extent the PE activities increase markedly as the amount of ammonium

tartrate in the medium is increased. The mycelial weight reaches a maximum at the 0·5 per cent. level, beyond which it remains fairly steady, but pectolytic activity continues to increase.

Apart from the medium with 0·1 per cent. ammonium tartrate, in which only slight growth occurred and in which there was no change in pH, the drift in all the media was from an initial 4·6–5·4 to a final 5·8. This alkaline drift may be due to the removal of pectic material or it may be due partly to the release of ammonium from the tartrate.

Replacement of 2 per cent. ammonium tartrate by equivalent (in terms of N) amounts of asparagine, glycine, ammonium chloride, or potassium nitrate gave inferior results in all cases. Growth was very poor on the last three media and all the tests for pectolytic enzymes were negative. Growth with asparagine was nearly as good as with ammonium tartrate but macerating and viscosity-reducing activities were much less, possibly because the reaction of the medium had moved to pH 8.

Media containing ammonium tartrate as source of N, which are suitable for production of pectolytic enzymes by *S. fructigena*, are likewise suitable for *B. cinerea*. Preparations from the latter fungus are in fact considerably more active. On the other hand, only weak preparations have been obtained from *S. laxa*. Comparable data have already been set out in Table III in another connexion.

Summarizing, one can state that an active pectolytic solution can be obtained by growing *S. fructigena* for a suitable period in a medium containing apple pectin, ammonium tartrate, and mineral salts. (It was later found that lemon pectin was equally suitable.) Maceration and viscosity activities are much greater in culture media which contain pectin than in those in which the pectin is replaced by glucose, thus indicating that the enzyme or enzymes measured are at least partially adaptive. There is a striking parallel behaviour of the maceration and viscosity activities, pointing to a close relationship of the enzymes involved. By increasing the quantity of nitrogen as ammonium tartrate to 2 per cent., a fairly steady increase in pectolytic activity resulted, although maximal growth was reached at the 0·5 per cent. ammonium tartrate level. With other nitrogenous sources, including asparagine, at concentrations of nitrogen equivalent to 2 per cent. ammonium tartrate, neither growth nor pectolytic activity was as good.

(*b*) *Apple extracts*

Fresh apple juice, prepared as described above (p. 18), was used as a culture medium in the following ways: at full strength or diluted with water; at full strength plus extra nitrogen in the form of ammonium tartrate, asparagine, or potassium nitrate at 1 per cent. concentrations; at full strength or diluted together with 0·5 per cent. ammonium tartrate, 0·1 per cent. KH_2PO_4, and 0·05 per cent. $MgSO_4.7H_2O$.

On all these media the growth of *S. fructigena* after 11 days at 25° was good, but little or no evidence of pectolytic enzymes was obtained. Only on diluted

apple juice (25 per cent., 10 per cent.) reinforced with ammonium tartrate and mineral salts was a trace of viscosity-reducing activity shown. Similar results were obtained for *S. laxa* and *B. cinerea*.

Since apple juice contains pectin, the medium reinforced with ammonium tartrate and mineral salts has all the requisite constituents for production of pectolytic enzymes, as the experiments with synthetic media have shown. It would seem therefore that there is some ingredient in apple extract which either prevents the formation of pectic enzymes or inactivates them after they have been formed. This would explain why, in the very dilute apple extracts only, was there any trace of enzyme activity, after growth of *S. fructigena*. This question will be taken up below (p. 32).

Effect of pH on Activity of Pectolytic Enzymes of *S. Fructigena*

The effect of pH on the pectolytic activity of culture extracts of *S. fructigena* was examined by the following methods: (*a*) maceration of potato discs 0·25 mm. thick, (*b*) viscometrically with different substrates, and (*c*) by measurement of the increase in reducing power of sodium pectate solutions. Preparations over the pH range 4–8 were set up with McIlvaine's buffer and pH determinations were with a pH meter.

Rate of maceration dropped from pH 7 to pH 8, but was uniform over the range 4–7. The curve obtained had thus a very broad and indistinct optimum.

The substrates used for viscometric determinations were sodium polypectate, apple pectin, and sodium pectate prepared from apple pectin as described above (p. 20). The viscosity activities expressed at V. 50 are recorded in Fig. 6.

With pectate and pectin an optimum at about pH 4·5 is shown, distinctly lower than the optimum (*c.* 6) for polypectate. Also the rate of attack on polypectate at its optimum is much greater than that on pectate or pectin at their optima. No explanation can be suggested for these differences as the exact chemical nature of the polypectate material is not known to the writer.

When PG catalyses the breakdown of pectin or pectic acid, an increase in reducing power results from the liberation of aldehydic groups of galacturonic acid or its polymers. The activity of an enzyme solution can be estimated by measuring this increase over a period of time. The method used for the determination of reducing power was in essentials that described by Jansen and MacDonnell (1945). The activity of an enzyme solution at a particular pH was recorded as the volume, in millilitres, of 0·1 N. iodine reduced in 5 hours. These volumes were calculated by deducting from the actual amount determined the volume of iodine reduced in the deactivated controls.

The activity of PG produced by *S. fructigena*, measured in this way with 1 per cent. sodium pectate as substrate, was greatest at pH 4·5 and fell off sharply between 5 and 7. This result closely resembles that obtained by measurement of loss of viscosity of apple pectin and sodium pectate (Fig. 6).

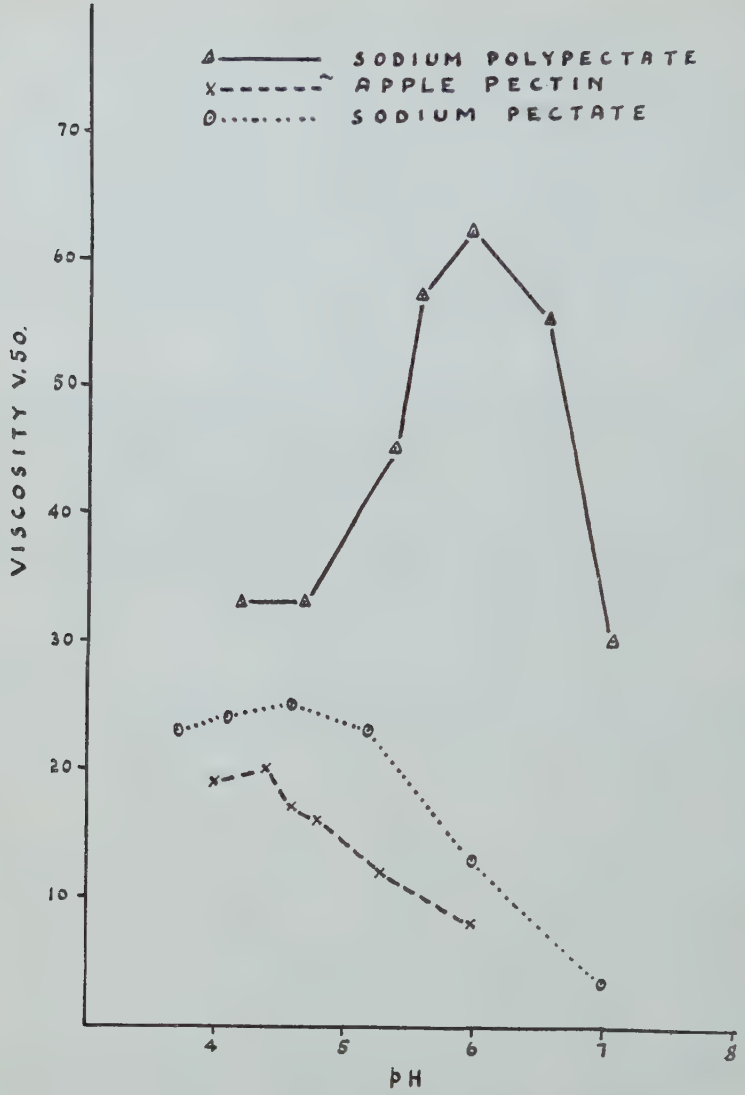

FIG. 6. Effect of pH on the viscosity-reducing activity of pectolytic enzymes of *S. fructigena* on pectic substrates.

As followed by the reducing method the rate of degradation of sodium pectate at 25° was linear for the first few hours, but by the 6th hour had become very slow. After 24 hours only 60 per cent. of the total possible reducing groups had been set free.

EFFECT OF PLANT MATERIALS ON ACTIVITY OF PECTOLYTIC ENZYMES

Evidence has been presented that pectolytic enzymes are produced by all the fungi studied on artificial culture media; also that pectic materials are

broken down in parasitized tissue. There is also evidence that apple extract media, although suitable for growth, show little or no pectolytic activity, either because the enzymes are not formed in these media or because they are inactivated after being formed. To test whether the latter was the correct explanation, the effect of apple extracts was estimated on active enzyme solutions from *B. cinerea* and *S. fructigena*. These enzyme solutions were obtained by growing *B. cinerea* on the medium described above (p. 25) and *S. fructigena* on a pectin-ammonium tartrate medium, also described in that section. *S. laxa* was not included in these tests because a really active pectolytic extract had not been obtained from this fungus.

As the hyphae of a parasitic fungus which causes rotting advance through the host tissue, the pectolytic enzymes, which are presumed to bring about rotting, are secreted and come into contact with juice of the host, liberated from the killed cells. Post-mortem oxidative reactions are likely to occur in such juices. It is possible therefore that those oxidative effects may act antagonistically to the pectolytic enzymes and may even tend to deactivate them. The following experiments were therefore devised to compare the effects on enzymic activity of plant juices which differed merely in respect of their oxidized or unoxidized condition. The oxidized type of extract is easily prepared; special precautions have to be adopted to prevent oxidation in the juice. The methods were as follows:

(i) *Oxidized juice.* Pre-frozen apples were thawed and minced; the minced tissue was allowed to stand, with frequent stirring, for several hours, then the juice was squeezed through muslin and further oxidized by bubbling air through it. It was cleared by centrifuging at 10,000 r.p.m. for 5 minutes. After this treatment it had a deep brown colour. Juices were similarly prepared from broad-bean leaves and potato tubers. These were black in the oxidized state.

(ii) *Unoxidized juice.* Oxidation was prevented by two methods. Pre-frozen apples were thawed at room temperature until it was just possible to squeeze the cold juice through muslin. It was added drop by drop into a beaker containing about 10 ml. of boiling distilled water which was kept boiling as more was added. 250 ml. were prepared at one time, so that the initial volume of water brought about a maximum of 4 per cent. dilution. In fact it was probably much less than this because of evaporation during the heating. Juice so prepared will be referred to as 'unoxidized (heat)' apple juice.

The other method made use of the fact that sodium cyanide is an inhibitor of copper oxidizing systems. A solution of sodium cyanide was added to freshly prepared cold juice from pre-frozen apples, so as to make the final concentration of cyanide $11{\cdot}6 \times 10^{-3}$ N. The degree of dilution in this case was 4 per cent. Juice prepared in this way will be referred to as 'unoxidized (cyanide)' apple juice. Both the unoxidized juices were cleared by centrifuging as before and were colourless.

To determine the effect of these juices on pectolytic enzymes they were

added to equal volumes of culture extracts of each of the fungi, left for 24 hours at room temperature under toluene, after which they were tested at pH 6·0 by maceration tests on potato discs 0·25 mm. thick, and viscometrically with sodium polypectate as the substrate. Activities were recorded in the usual way.

Table VI gives the results obtained with enzyme solutions of *B. cinerea* set up in the variety of ways shown. The concentrations of apple juice and enzyme were brought to the same level in all the solutions by the addition of distilled water to compensate for the sodium cyanide solution added. Sodium cyanide when present was at a concentration of $5{\cdot}8 \times 10^{-3}$ N.

Before considering the data in Table VI it should be noted that oxidizing

TABLE VI

Effect of Apple Juices on Pectolytic Enzymes of B. cinerea

	Viscosity V. 50	Maceration	Colour on mixing	Colour after 24 hrs.
(*a*) Oxidized juice	2	0·2	Brown	Dark brown
(*b*) ,, ,, +cyanide	23	1·0	,,	Brown
(*c*) Unoxidized (heat)	9	0·2	Colourless	,,
(*d*) ,, ,, +cyanide	110	4·0	,,	Colourless
(*e*) ,, (cyanide)	110	4·0	,,	,,
(*f*) Water	110	4·0	,,	,,
(*g*) Cyanide	110	4·0	,,	,,

systems are present both in extracts of apple cells and in preparations of *B. cinerea* enzyme made from ordinary synthetic media. *B. cinerea* culture solutions give a positive reaction with the catechol test, indicating the presence of a polyphenolic oxidase enzyme. Two millilitres of enzyme solution were added to 2 ml. of N/5 catechol at pH 5·0. The solution turned brown in about 30 minutes, whereas controls in which boiled enzyme solution was used remained colourless.

Unoxidized (heat) apple juice, which is colourless, was mixed with *B. cinerea* enzyme and left in a test-tube under toluene. It became brown overnight, while a control in which boiled enzyme was used remained colourless. Sodium cyanide in sufficient quantity prevented the oxidation of the enzyme-apple juice mixture.

Oxidizing enzymes are also present in apple (Hackney, 1948) and are responsible for the browning which accompanies wounding of the tissue, and this was confirmed with the catechol test.

Table VI shows the following points:

(i) By comparison of (*f*) and (*g*) it is seen that cyanide at the concentration used did not affect the rate of pectolytic action.

(ii) All the preparations in which oxidase action did not occur, viz. (*d*), (*e*), (*f*), and (*g*), showed high pectolytic activity as against low activity in (*a*), (*b*), and (*c*) where oxidation took place.

(iii) In (*a*) oxidation was due in the first instance to active oxidase derived from apple juice, but was intensified by oxidase present in the *Botrytis* preparation. In (*b*) the fungal oxidase was prevented from acting as cyanide was added at the time of mixing. In (*c*) oxidation is due to the fungal oxidase after mixing. Preparation (*c*) was almost as completely deactivated as (*a*); (*b*) was still fairly active.

TABLE VII

Effect of Time on Pectolytic Activity of an Enzyme Solution of B. cinerea *diluted with an Equal Volume of Oxidized Apple Juice*

Time (hrs.)	Viscosity (V. 50)	
	Dilution with water	Dilution with apple juice
1·5	140	125
6	140	18
24	140	<2

Table VII shows the effect of time on the pectolytic activity of a preparation of *B. cinerea* diluted with an equal volume of (*a*) water, (*b*) oxidized apple juice. Whereas the former maintains its activity, the latter is considerably weakened in 6 hours and is almost completely deactivated in 24 hours.

In an attempt to separate the portion of oxidized apple juice which contained the inactivating principle, solutions were prepared containing: (i) the portion of oxidized apple juice insoluble in 63 per cent. ethyl alcohol, prepared by adding 100 ml. of 95 per cent. ethyl alcohol to 50 ml. of juice, leaving it

TABLE VIII

Effect of Various Fractions of Oxidized Juice on Pectolytic Enzymes of B. cinerea

	Viscosity (V. 75)	Maceration
Water	100	5·0
Oxidized apple juice	<2	0·4
(i) Alcohol insoluble portion	100	4·5
(ii) Alcohol soluble portion	<2	0·4
(iii) Dialysed soluble portion	90	3·7

to stand overnight, filtering, washing precipitate with alcohol and redissolving it in 50 ml. of water; (ii) the portion soluble in 63 per cent. ethyl alcohol, prepared by concentrating the filtrate in (i) under reduced pressure until no trace of alcohol could be detected (the final volume was 45 ml.); (iii) solution (ii) after dialysis through a cellophane membrane, against distilled water, for 64 hours. These solutions were mixed with equal volumes of *B. cinerea* enzyme solution, left for 24 hours at room temperature under toluene, and tested for pectolytic activity in the usual way. Table VIII gives the results which show that the inactivating principle is soluble in 63 per cent. ethyl alcohol and can be removed from this solution by dialysis.

The effect of apple juice, oxidized or unoxidized, on pectolytic activity of *S. fructigena* preparations was less marked than in the case of *B. cinerea* and was only given preliminary examination. Under conditions where the activity of *Botrytis* preparations was almost destroyed, the activity of *S. fructigena* preparations was reduced to about half, measured viscometrically or by the maceration technique.

In further preliminary tests with oxidized juice of broad-bean leaf and of potato tuber, the former gave a definite repressing action on enzymic preparations of *B. cinerea* and *S. fructigena*, the latter a small and uncertain effect. Parallel tests with unoxidized extracts were not carried out, so that it is possible that salts present in the plant extracts were responsible for the effects observed.

It should also be mentioned that the writer found, towards the end of this work, that the pectolytic enzymes from a different strain of *B. cinerea*, originally isolated from lettuce, were not inactivated by oxidized apple juice. The activities were measured viscometrically. No further work was done in this connexion, but the main results recorded in this section were repeated and confirmed with the strain of *B. cinerea* originally used, which was isolated by the writer from apple.

Discussion

The experiments described in this paper go a long way towards clearing up what seems to be anomalous behaviour on the part of *Sclerotinia fructigena* and *Botrytis cinerea* as parasites of apple fruit. The position may now be outlined as follows.

Both these fungi (and *S. laxa*), as was shown above (p. 24), destroy pectic material, soluble and insoluble, in attacked fruits. As regards structural damage to the host tissue, the loss of insoluble pectic substance is of the greater significance, and it is noteworthy that the effect of *S. fructigena* in this connexion is considerably less than that of the other two fungi (Table IV). It can be plausibly suggested that the more pronounced rotting of the apple tissue which is associated with attack by *S. laxa* and *B. cinerea* is to be explained in this way. The fact that pectic substances are destroyed in the tissue by all three fungi points indubitably to the excretion of pectolytic enzymes by the advancing hyphae, as no substances other than these enzymes can be suggested in explanation of the observed changes.

Microscopic examination in the region of invading hyphae showed that all three fungi bring about death of the host cells in their neighbourhood. The fungal hyphae are therefore embedded in dead tissue, in which the normal post-mortem reactions characteristic of that tissue will take place. Prominent among these is the oxidative reaction, leading to a brown coloration, which arises by the mixing of a protoplasmic oxidase with a vacuolar oxidizable substance in the presence of an oxygen supply.

In cultures on suitable synthetic media all three fungi were shown to pro-

duce pectolytic enzymes, though in unequal degree (Table III). The most active preparations were obtained from *B. cinerea*, *S. fructigena* being next, and *S. laxa* last. Nevertheless little or no enzyme was obtained from extracts of parasitized apple fruit or from culture filtrates on apple decoctions. Furthermore, when an apple decoction was reinforced with synthetic ingredients which in themselves were known to give active enzymic preparations, again little evidence of pectolytic enzymes was obtained. It was clear therefore that the presence of apple juice in the culture medium either prevented the secretion of pectolytic enzymes or brought about their deactivation sufficiently rapidly to make their detection difficult.

It was shown above (p. 35) that active pectolytic preparations of *B. cinerea* were rapidly deactivated in presence of oxidized apple juice. Almost complete deactivation had occurred in 24 hours, whereas there was no loss of activity in the same time in the presence of juice which was prevented from becoming oxidized. This effect was most pronounced with *B. cinerea* enzyme; it was also shown, but less markedly, with *S. fructigena* enzyme.

In the light of these results there is no difficulty in reconciling the fact that pectolytic action has taken place in parasitized apple tissue with general failure to demonstrate the presence of pectolytic enzymes in extracts of that tissue. As was pointed out above (p. 17), pectolytic activity has been proved for mycelial extracts and it is highly probable that enzymic secretion and excretion are most active at the growing ends of hyphae (Brown, 1917). The enzymes so excreted at the advancing surface of the lesion remain active sufficiently long to bring about the pectolytic effects, but the process of deactivation goes on all the time, so that a negligible amount of enzyme is present in the lesion as a whole.

Any attempt at present to explain the characteristic differences between *Botrytis* attack and *S. fructigena* attack on apple fruit would be premature or at best conjectural. One does not, for example, understand why the latter is much the more active parasite at ordinary temperature (*v.* Table I), as regards both speed of invasion and ability to attack unripe fruit. The more pronounced rotting effect shown by *B. cinerea* may be a direct outcome of its slower rate of progression, the growing hyphal margin remaining for a longer time at one place so that a more complete maceration is effected. Or it may be that there is much less excretion of pectolytic enzymes by *S. fructigena*, and though the latter are not so prone to deactivation as those of *B. cinerea*, the total effect on the cell walls is in fact less with *S. fructigena*.

It has long been held that parasitic invasion, by killing the host cells, may lead to the formation of substances not originally present—e.g. tannins, phenolic substances—and that these may contribute to arresting further progress by the parasite. The work of this paper shows that another type of reaction comes into play, viz. an interaction between oxidizing and pectolytic systems, and though in the two cases examined the ultimate effect is not to halt invasion, it is quite possible that in others it may do so. Obviously a considerable field lies open for investigation.

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Experimental and Analytical Studies of Pteridophytes

XXXI. The Effect of Shallow Incisions on Organogenesis in *Dryopteris aristata* Druce

BY

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With Plates I and II and thirty Figures in the Text

Abstract

The hypothesis, that the regulative effect of the shoot apex and adjacent leaf primordia on incipient growth centres is exercised by way of the incipient or pre-vascular tissue, has been tested by isolating leaf primordia and primordium sites, and also the shoot apex, by various shallow incisions. These incisions were such as to pass through the single superficial layer of meristem cells of the shoot apex, the incipient vascular tissue being left intact. Young primordia thus isolated by tangential incisions developed normally as leaves whereas with deep incisions buds are usually induced. Primordia isolated by four shallow incisions when the apex was not in active growth usually became dispersed and disappeared; the inception of prospective primordia could also be prevented in this way. The hypothesis relating to the functional activity of the pre-vascular tissue in morphogenesis, which is supported by the results obtained, is discussed in the light of other recent experimental evidence.

Introduction

THE shoot apex of *Dryopteris aristata* Druce (*D. austriaca* (Jacq.) Woynar) consists of three fairly well-defined tissue layers—the superficial and conspicuous prism-shaped cells of the apical meristem, the underlying smaller-celled incipient vascular (or pre-vascular) tissue, and the central pith (Text-fig. 1). In experimental investigations on this fern it has been shown that if the I_1 position (i.e. the position of the next leaf primordium to be formed), or a visible young leaf primordium which has not yet developed a lenticular apical cell, is isolated from the shoot apex by a wide and deep tangential incision, it will develop as a solenostelic bud (Wardlaw, 1949*a*; Cutter, 1954). It has also been shown that young leaf primordia isolated by deep radial incisions rapidly outgrow the adjacent older primordia (Wardlaw, 1949*a*). In all these experiments the incisions were such as to sever the incipient vascular tissue and penetrate the pith.

These experiments support the concept of growth centres and fields as applied to the fern shoot apex (Wardlaw, 1949*a*), and indicate that a regulative effect is exercised upon leaf and bud sites and upon young developing primordia by the shoot apex and the older leaf primordia. A consideration of the data suggested that this regulative effect might be exercised by way of the incipient vascular tissue. If so, shallow incisions which did not penetrate and sever the incipient vascular tissue should not induce any comparable organogenic

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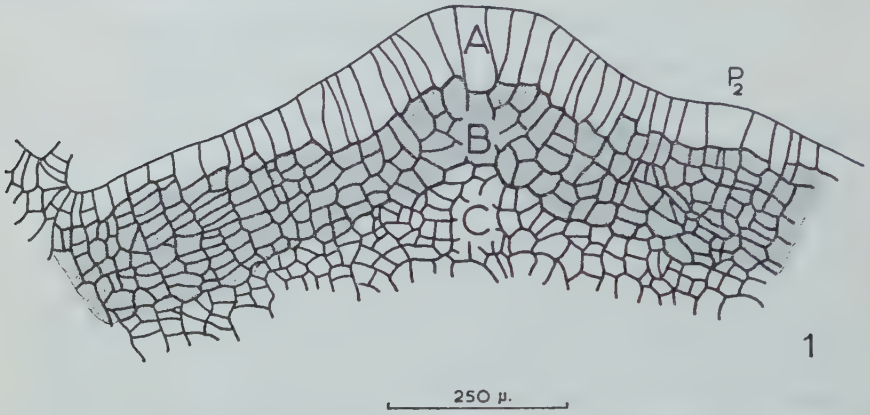
effect. This hypothesis has been tested experimentally by observing the effect of shallow incisions on growth and development at the apex of *Dryopteris aristata*. A brief comparison of the effect of deep and shallow incisions on organogenesis in this fern has already been published (Wardlaw and Cutter, 1954): the relevant experiments are now more fully described and discussed.



TEXT-FIG. 1. Longitudinal section of the shoot apex showing the disposition of the tissues. A, apical meristem; B, incipient vascular tissue; C, pith. (×95.)

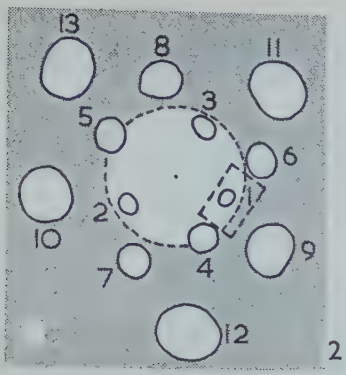
MATERIALS AND METHODS

Large apices of *Dryopteris aristata* were laid bare as described in earlier papers (Wardlaw, 1944*b*) and maintained in pans of peat in the laboratory. During the winter season specimens were sometimes transferred to a room maintained at a constant temperature of $21 \pm 1°$ C. By using very fine knives it was found possible to make very shallow incisions: these penetrated only a single layer of cells (*see* Anatomical Observations). The positions of the incisions are indicated diagrammatically in Text-figs. 2–7. The terminology for leaf sites and primordia is that now generally used in studies of phyllotaxis (Snow and Snow, 1931).

EXPERIMENTAL RESULTS

(*a*) *Isolation of leaf primordia and primordium sites by four shallow incisions*

Leaf primordia and primordium sites were isolated within large rectangles by four shallow incisions as in Text-fig. 2. The majority of primordia isolated were undetermined P_1 primordia, i.e. not yet possessing a lenticular apical cell. When similarly isolated but by deep incisions such primordia usually develop as buds (Cutter, 1954). A few older primordia, already determined as leaves, and I_1 positions were also isolated by shallow incisions. The results are presented in Table I. In each series a few control specimens were included in which the primordium or site was isolated by deep incisions; without exception these yielded buds.



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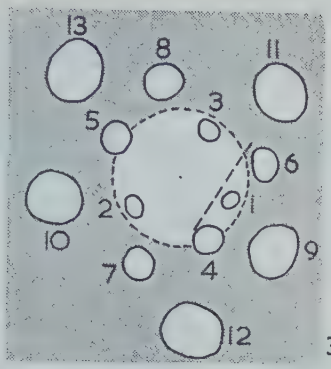

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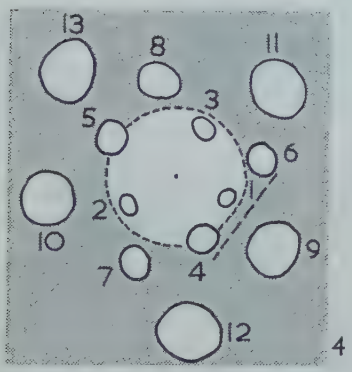

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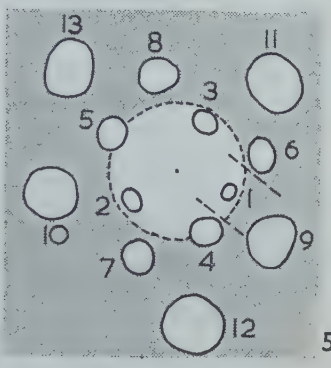

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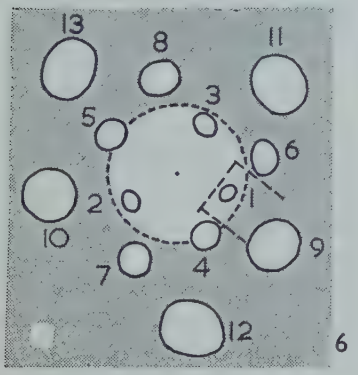

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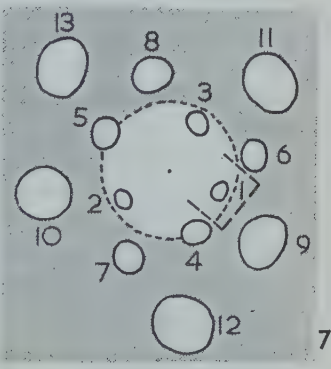

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TEXT-FIGS. 2–7. Diagrams showing the position of the incisions.
TEXT-FIG. 2. Four incisions.
TEXT-FIG. 3. Single adaxial incision.
TEXT-FIG. 4. Single abaxial incision.
TEXT-FIG. 5. Radial incisions.
TEXT-FIG. 6. Adaxial and radial incisions.
TEXT-FIG. 7. Abaxial and radial incisions.

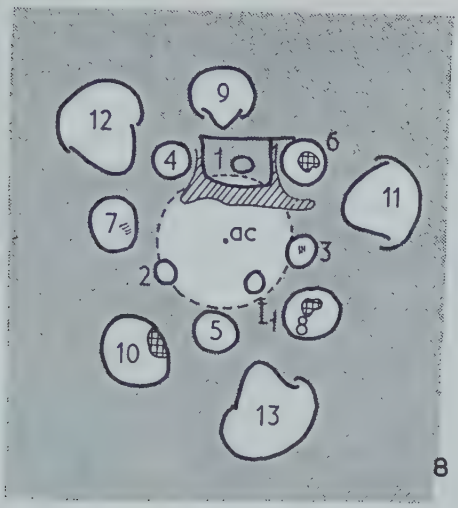
TABLE I

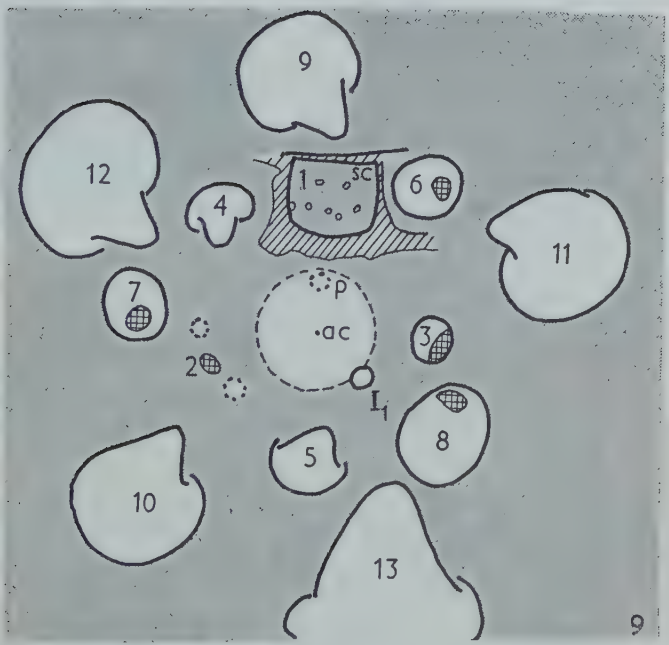
Isolation of Leaf Primordia and Primordium Sites by four Shallow Incisions

Primordium isolated	Total no. isolated	No. which developed as buds	No. which developed as leaves	No. which disappeared or were not formed
I_1	8	—	5	3
P_1	44	1*	16	27
P_2	4	—	4	—
P_3	2	—	2	—
P_4	1	—	1	—

* As this specimen had formed no new primordia at the end of a month, it may be inferred that the apex was in an inactive condition.

It is evident that, in contrast to the effect of deep incisions, shallow incisions do not induce isolated primordia or sites to develop as buds. Primordia which were not determined as leaves at the time of isolation either continued to develop as leaves or ceased growth and gradually disappeared. This unexpected result was carefully verified. Isolated P_1 primordia which eventually disappeared could be observed on the isolated plugs during the first two or three weeks after isolation (Pl. I, Fig. 1), but after about three weeks they were no longer in evidence (Pl. I, Fig. 2; Text-figs. 8–12). At this stage the isolated plug usually became somewhat concave, and scales were formed on it, including the P_1 site, i.e. the latter had become parenchymatous. Similarly isolated I_1 positions underwent a comparable histological development, the primordia failing to appear at all (Table I); but the untreated sites of I_2, I_3, &c., in due course gave rise to normal leaf primordia (Text-figs. 12–14). (All specimens in which the I_1 position might have been injured by the adaxial cut have been excluded from Table I). In those experimentally isolated primordia which had already been determined as leaves, i.e. P_2–P_4, the further development was normal. Some of the isolated P_1 primordia continued to grow and developed as leaves; two of these became larger than normal. Three isolated primordia, which at first continued their development as leaf primordia, ultimately became scaly parenchymatous mounds. The majority of primordia which continued to develop were isolated during the season of rapid growth. This suggested that a seasonal factor was involved, i.e. that on actively growing apices isolated primordia were able to continue their growth, whereas on less actively growing apices they were unable to do so and eventually disappeared. An additional experiment was therefore carried out in which apices were laid bare and were thereafter allowed to grow on for three weeks; at the end of this time the youngest visible primordium was isolated by four shallow incisions. Of six specimens treated in this way, all the isolated primordia continued to grow and develop as leaves (Text-fig. 15), even when the cuts were very close to the primordium (Pl. II, Fig. 7). The growth rate and reactivity of the apex thus appear to be important in determining the further development of primordia isolated by shallow incisions.



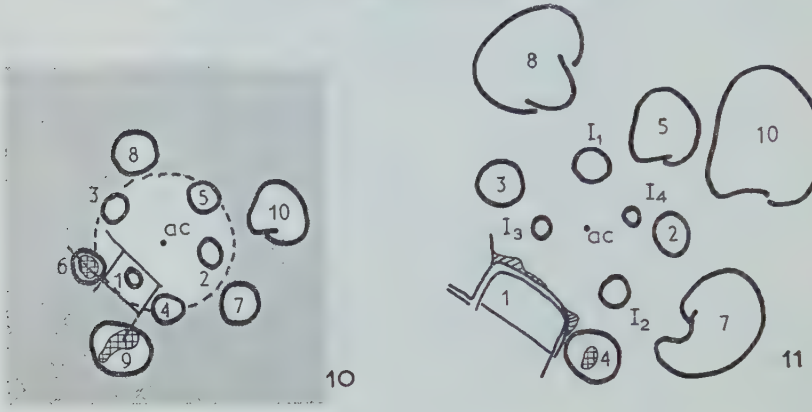


TEXT-FIGS. 8–9. An apex in which P_1 was isolated by four shallow incisions. (Compare Pl. I, Figs. 1 and 2.)

TEXT-FIG. 8. Twenty-three days after isolation of P_1. P_1 is still quite visible on the isolated plug, and I_1 is present.

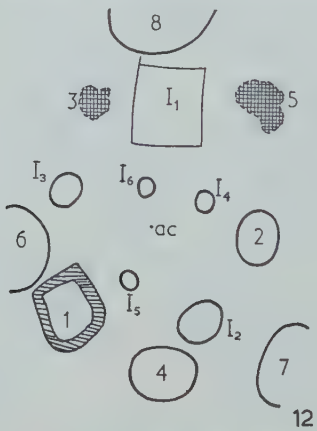
TEXT-FIG. 9. Sixteen days later. P_1 has disappeared, and scales have arisen on the isolated plug of tissue. (×18.) *p*—the second primordium to arise—considerably displaced from the I_2 position.

In specimens in which the shoot tip has been mechanically damaged either by puncturing or fortuitously in the course of the experimental treatment, primordia isolated by four shallow incisions developed either as leaves or as buds. Some I_1 sites did not give rise to primordia, but in a few instances this



Text-figs. 10–11. An apex in which P_1 was isolated by four shallow incisions.
Text-fig. 10. One day after isolation of P_1.
Text-fig. 11. Twenty-eight days after isolation of P_1. P_1 is no longer visible upon the isolated plug, but four new leaf primordia have arisen on the apex. (×14.)

may have been due to the proximity of the cuts. It is clear, therefore, that when the regulative effect of the apical cell region is removed, primordia isolated by four shallow cuts are capable of growth, and may develop as buds.



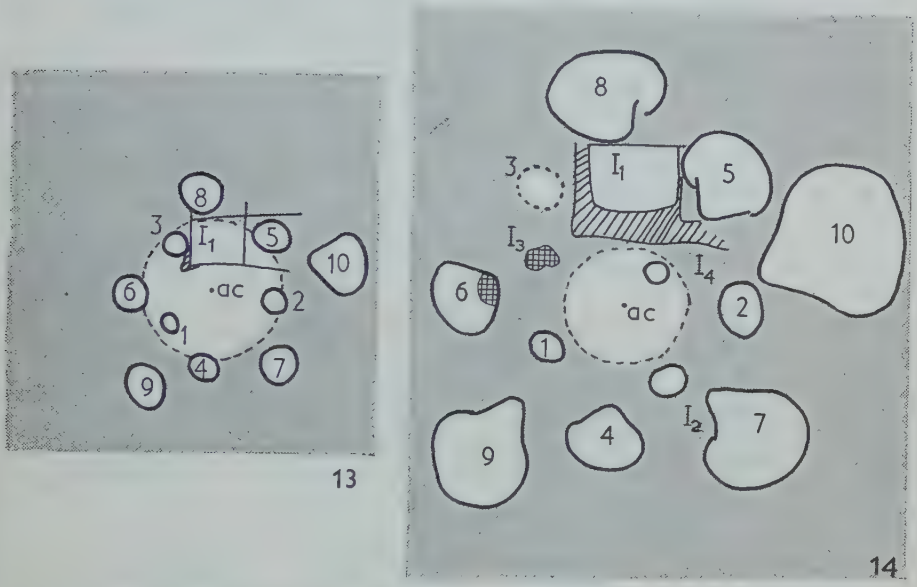
Text-fig. 12. A specimen in which P_1 and the I_1 position were each isolated by four shallow incisions. P_1 has ceased growth and disappeared and I_1 has not appeared. Five new primordia have arisen on the apex. Forty-three days after isolation. (×14.)

Experiments to ascertain the effect of a subsequent deepening of the adaxial incision, i.e. after the disappearance of the isolated primordium, are in progress: preliminary results show that after a certain time has elapsed the capacity of the primordium for meristematic growth is entirely lost.

The results described in this section are in sharp contrast to those obtained by isolating comparable primordia by deep incisions. They demonstrate that, if the apical cell group is intact, its regulative action on primordia isolated by shallow incisions, i.e. not severing the incipient vascular tissue, is still effective.

(*b*) *Isolation of primordia and primordium sites by other systems of shallow incisions*

Leaf primordia and presumptive leaf positions were also isolated by single adaxial or abaxial and by radial incisions, and by combinations of these (Table



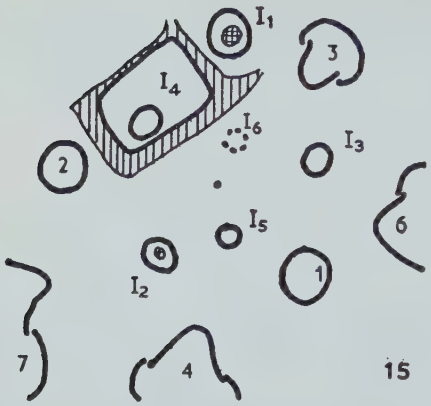
TEXT-FIGS. 13–14. An apex in which the presumptive position of I_1 wasated by four soil shallow incisions.

TEXT-FIG. 13. On the day the incisions were made.

TEXT-FIG. 14. Thirty-seven days later. No primordium has appeared at I_1, but I_2–I_4 have been formed. (×18.)

II and Text-figs. 3–7). Both deep and shallow abaxial incisions were without effect on organogenesis, all the primordia isolated developing as normal, dorsiventral leaf primordia. Shallow radial incisions were also without effect (Text-fig. 16), and there was no evidence of the rapid and extensive growth resulting from similar deep cuts (Wardlaw, 1949*a*). A combination of radial and abaxial incisions, both deep and shallow, was also ineffectual (Text-figs. 17 and 21).

Primordia isolated by a shallow adaxial incision usually continued to develop as leaves (Text-fig. 18), but a few P_1 primordia disappeared and some I_1 sites failed to yield primordia (Table II). Similarly, a combination of shallow radial and adaxial incisions was capable of causing the disappearance of isolated primordia (Text-figs. 19 and 21), although the majority of primordia



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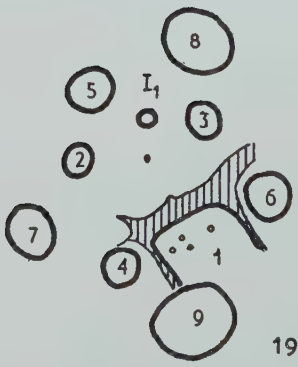

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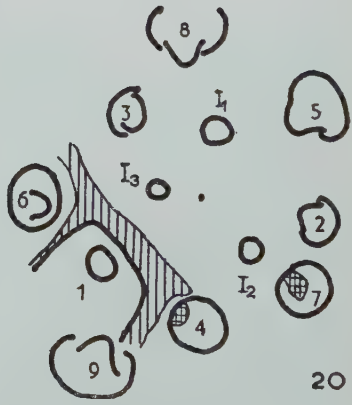

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TEXT-FIGS. 15–20. Specimens in which primordium P_1 was isolated by various systems of shallow incisions.

TEXT-FIG. 15. This apex was laid bare and allowed to grow on for three weeks. At the end of that time four new leaf primordia had been formed, and the youngest visible leaf primordium (I_4) was isolated by four shallow incisions. Seventeen days later, as shown here, I_4 was developing as a normal leaf primordium and two further primordia had appeared.

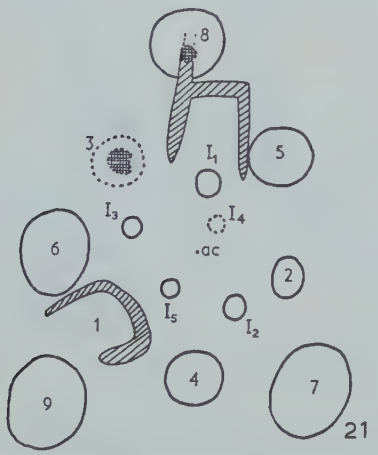
[*cont. on opposite page*]

TABLE II

Experimental Isolations of Leaf Primordia and Primordium Sites by Various Shallow Incisions

Position and number of the cuts	Primordium isolated	Total no. of isolations	No. which developed as buds	No. which developed as leaves	No. which disappeared or did not arise
Adaxial (1)	I_1	8	—	5	3
	P_1	11	—	9	2
Abaxial (1)	I_1	6	—	6	—
	P_1	6	—	6	—
	P_2	2	—	2	—
	P_3	2	—	2	—
Radial (2)	I_1	6	—	6	—
	P_1	9	—	9	—
Adaxial and radial (3)	I_1	4	—	4	—
	P_1	16	1	10	5
Abaxial and radial (3)	I_1	8	—	8	—
	P_1	10	1*	9	—

* Slight fungal infection on the apex.



TEXT-FIG. 21. A specimen in which P_1 was isolated by shallow adaxial and radial cuts, and the I_1 position by shallow abaxial and radial cuts. P_1 has ceased growth and disappeared, while I_1 has developed normally; I_2–I_5 also present. Twenty-six days after isolation. (×14.)

TEXT-FIG. 16. An apex in which P_1, isolated by shallow radial incisions, has developed as a leaf primordium of normal size. Twenty-four days after isolation of P_1.

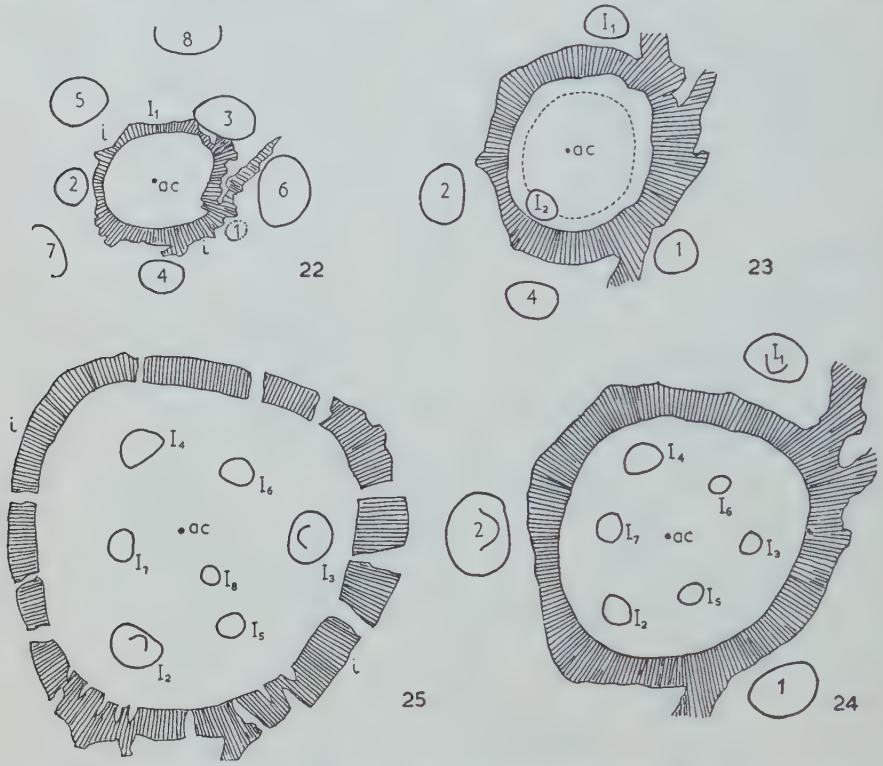
TEXT-FIG. 17. P_1, which was isolated by abaxial and radial shallow incisions, has developed as a normal leaf primordium. Twenty-five days after isolation of P_1.

TEXT-FIG. 18. A specimen in which P_1, isolated by a shallow adaxial incision, developed as a normal leaf primordium. Twenty-eight days after isolation of P_1.

TEXT-FIG. 19. An apex in which P_1, isolated by shallow adaxial and radial cuts, ceased growth and disappeared. Scales have arisen at its position. I_1 has arisen on the apex. Twenty-five days after isolation of P_1.

TEXT-FIG. 20. An apex in which P_1 was isolated by shallow adaxial and radial cuts, and continued to grow and develop as a normal leaf primordium. Thirty-six days after isolation of P_1. All (×14.)

thus isolated continued normal growth (Text-fig. 20). Comparable primordia and leaf sites isolated by deep adaxial incisions develop as buds (Wardlaw, 1949*a*; Cutter, 1954). A comparison of the records in Tables I and II shows that a higher proportion of primordia isolated by four shallow incisions cease



TEXT-FIGS. 22–25. A specimen in which the apical cone was isolated from the surrounding leaf primordia by shallow incisions.

TEXT-FIG. 22. Five days after isolation.

TEXT-FIG. 23. Twenty-five days later. Parenchymatization has taken place around the wound. The apical cone has enlarged considerably, and I_1 and I_2 have arisen.

TEXT-FIG. 24. Eleven days later. Five new leaf primordia have been formed in rapid succession.

TEXT-FIG. 25. Eight days later (49 days after isolation). The isolated apical cone has undergone considerable enlargement, and eight new primordia have been formed since the beginning of the experiment. *i–i*, incisions. (× 14.)

growth and disappear than those isolated by any other system of incisions, and also that an adaxial cut has a greater effect in this respect than an abaxial one. Primordia isolated by these various shallow incisions are capable of continued growth and may develop as buds if the shoot tip is damaged.

(*c*) *Isolation of the shoot apex by shallow incisions*

Earlier experiments have shown that an apical meristem of *Dryopteris* which has been isolated from adjacent tissues by four deep incisions is capable

of continued axial growth with the formation of leaf primordia (Wardlaw, 1945, 1947, 1949*b*). Under these conditions the isolated lateral region typically gives rise to large solenostelic buds. When the incisions were relatively close to the shoot tip, the apical meristem grew slowly and became considerably reduced in size and was soon outgrown by the lateral buds (Wardlaw, 1949*b*). When, however, shoot apices were isolated by shallow incisions, buds were not formed on the tissue outside the cuts and the isolated apex continued to grow actively (Text-figs. 22–25). In the specimen illustrated, there was a lag in the appearance of I_1 and I_2, associated with enlargement of the wound and adjacent parenchymatization. It will be seen, however, that there was considerable growth of the apical region enclosed by the cuts (Text-figs. 22 and 23). Subsequently, primordia were formed in rapid succession, and the isolated area grew rapidly (Text-figs. 24 and 25).

Anatomical Observations

To ascertain the depth of the cuts, apices in which leaf primordia had been isolated by shallow adaxial incisions were sectioned longitudinally at intervals. As can be seen in Pl. II, Figs. 3–5, the cut penetrated only the superficial layer

Text-fig. 26. Longitudinal section of a specimen in which P_1, isolated by shallow adaxial and radial incisions, ceased growth and disappeared. A conspicuous ridge of seriate wound tissue (w.t.) has been stimulated by the adaxial cut. Incipient scales (sc.) are present at the position of P_1. (Suberized tissue cross-hatched). (×95.)

of cells of the apical meristem, the incipient vascular tissue remaining intact. Differential staining indicates that cells adjacent to those destroyed by the cut may have been affected to some extent (Pl. II, Figs. 4 and 5). The injured tissue eventually becomes brown (Pl. II, Fig. 5). After some time there is a conspicuous development of wound tissue, especially beneath the adaxial and radial incisions (Pl. II, Fig. 6); (Text-fig. 26). This consists of cambiform files of cells which may be continuous with those of the incipient vascular tissue. The adaxial cuts eventually become raised up on a mass of wound tissue.

TEXT-FIGS. 27–28. Basipetal serial sections of an apex in which P_1 disappeared after isolation by four shallow incisions. There is no leaf-trace or leaf-gap associated with P_1, although the leaf-trace of I_1 is evident. P_9 was damaged. (Incipient vascular tissue outlined with a broken line). (×20.)

TEXT-FIGS. 29–30. Basipetal serial sections of an apex in which P_1 disappeared as a result of isolation by four shallow incisions and the adaxial cut was subsequently deepened. No growth of the isolated plug took place, however, indicating that the capacity of P_1 for meristematic growth had been entirely lost. No leaf-trace or leaf-gap is associated with P_1, although those associated with I_1 are evident. (*i–i*, deepened adaxial incision; necrosed tissue resulting from this dotted; incipient vascular tissue outlined with a broken line). (×20.)

Comparatively little wound tissue is formed in relation to the abaxial incisions (Pl. II, Fig. 6).

In longitudinal radial sections of the position of a primordium which has disappeared as a result of the experimental treatment, it can be seen that there is no leaf-trace associated with the isolated primordium (Pl. II, Fig. 6), although incipient vascular tissue is present in proximity, whereas conspicuous leaf-traces are associated with those isolated primordia which have continued to grow and develop (Pl. II, Fig. 7). A primordium, isolated by four shallow incisions, which initially grew and developed as a normal leaf primordium, but which subsequently became parenchymatous and gave rise to scales, is illustrated in Pl. II, Fig. 8. Its vascular system proved to be of particular interest in that a leaf-trace was present basally which made normal contact with the shoot stele but faded out near the parenchymatous tip of the primordium. That isolated primordia which have disappeared have no associated leaf-traces or leaf-gaps has been confirmed in transverse sections of apices (Text-figs. 27 and 28). In primordium isolation experiments, in which the primordium had disappeared and in which the adaxial cut was subsequently deepened, but where no organ development was induced, leaf-traces and leaf-gaps are also absent (Text-figs. 29 and 30).

These observations support the hypothesis that a substance diffusing from an active growth centre is involved in the formation of incipient vascular tissue (Wardlaw, 1944*a*); i.e. when a growth centre loses its capacity for meristematic activity no associated incipient vascular tissue is formed. Young (1954) has recently demonstrated experimentally that the presence of a leaf primordium is necessary for the differentiation of its leaf-trace, and that while auxin is necessary for the maintenance of the cells in a meristematic condition, some other substance, derived from the primordium, is necessary for the differentiation of procambium.

Discussion

The hypothesis under consideration, that is, that the regulative action of the apical cell group and adjacent leaf primordia takes place by way of the pre-vascular, or incipient, vascular tissue, is supported by the results obtained. The severance of the pre-vascular tissue leads to important changes in the normal organogenic activity of the apex. Shallow incisions, by contrast, yield no comparable results.

The transformation of a young leaf primordium, i.e. an organ of dorsiventral symmetry and ultimately limited growth, into a bud, i.e. an organ of radial symmetry and potentially unlimited growth, by a wide and deep tangential incision is of course a remarkable phenomenon. The suggestion has already been made that the characteristic symmetry and orientation of the leaf, with its limited adaxial growth and extensive tangential and abaxial growth, may be due to the position in which it originates, that is, on the side of the conical apex, together with inhibitional effects proceeding from the apical cell group (Wardlaw, 1949*a*).

Recent investigations have indicated that over-simplified explanations of organogenesis in terms of the effects of growth-regulating substances, originating in the shoot apex and lateral growth centres, are likely to be inadequate. Thus it has been found that young leaf primordia and I_1 positions, partially isolated from the apical cell group by two deep adaxial incisions which did not meet, were sometimes capable of developing as buds, even though an intact bridge of tissue, including incipient vascular tissue, remained between the primordium and the apex (Wardlaw, 1955*a*, 1955*b*). The results of these experiments, and of others on the effect of puncturing the apical cell (Wardlaw and Cutter, 1955), support the view that the action of the distal region of the apex on organogenesis is not a simple, direct one of the kind suggested above, but that it is mediated through the growth and organization of the apex as a whole. Thus, a deep adaxial incision will not only preclude the basipetal movement of possible growth-inhibiting substances from the apical cell group: it will interfere radically with the whole system of metabolic gradients in the sector of the incision, the more so as a rapid and extensive wound-reaction is a conspicuous feature of injured meristems. Sussex (1952) considers that competition for nutrients may be an important factor in maintaining the form of the apical meristem; and Skoog (1950) has emphasized the importance in morphogenesis of the balance between growth-regulating substances and nutrients. The present investigations suggest that the incipient vascular tissue may constitute a channel for the rapid translocation of these substances. This finding is supported to some extent by the observations of Ball (1952) on isolated meristems of *Lupinus albus*. He found that the buds which arose on the lateral segments were inhibited by the shoot apex only after contact had been established between the stele of the isolated shoot and the original vascular tissue of the axis.

That the inception of primordia may be prevented and that the disappearance of existing primordia may be brought about by isolation of the area by four shallow incisions is a remarkable and unexpected finding. The results presented in Table II indicate that of these four cuts the adaxial one is the most effective; yet the majority of primordia isolated from the shoot apex by a single shallow adaxial incision continue to grow and develop as leaves. An interpretation based on the hypothesis that a substance essential for the inception and growth of leaf primordia is transmitted in the superficial layer of the shoot apex would therefore prove inadequate. It has been suggested that the conspicuous formation of wound tissue, associated in particular with the adaxial and radial incisions, might be related to the disappearance of the isolated primordium (Wardlaw and Cutter, 1954). This tissue might conceivably compete for the nutrients and other substances necessary for meristematic growth and thus bring about the dissipation of the primordium. Since I_1, P_1, or P_2, isolated by deep cuts during the same period, do grow, it may be inferred that the isolated panel has enough of the substance(s) required for organ formation, provided the inhibitive or regulative effects of the apical cell group and adjacent leaf primordia are eliminated. The possibility that an

auxin-inactivation system might be operative at the cut surfaces, as is known to occur in other ferns (Steeves, Morel, and Wetmore, 1953), cannot be overlooked.

The apparent correlation between the growth rate of the apices and the development of primordia isolated by shallow incisions emphasizes the importance of the general reactivity of the individual apex in studies of morphogenesis. Because of this, experimental results are sometimes variable and difficult to interpret.

These findings make available a method for preventing the inception of prospective primordia, or for the elimination of meristematic potentiality in incipient primordia, without injury to the primordium itself. This may afford a useful tool for future investigations of morphogenesis.

Summary

1. The hypothesis that, in the apex of *Dryopteris*, the regulative effect of existing growth centres upon the formation and development of new ones is exercised by way of the incipient vascular tissue has been tested experimentally.

2. Leaf primordia and primordium sites were isolated from the shoot apex and/or adjacent leaf primordia by various systems of shallow incisions, so that the incipient vascular tissue remained intact.

3. In apices which were in a state of active growth, leaf primordia isolated by four shallow incisions continued to grow and develop as leaves, whereas with comparable deep incisions buds were usually obtained. Under conditions of less active growth, primordia isolated by shallow incisions usually disappeared and the inception of prospective primordia was prevented.

4. Single adaxial incisions, and a combination of an adaxial and two radial incisions, usually had no effect on the isolated primordia, but a proportion of these primordia also disappeared.

5. Shallow radial or abaxial incisions, or a combination of both, had no effect on the growth or development of the isolated primordia.

6. When the apical cell group was damaged, primordia isolated in any of these ways were capable of growth and frequently developed as buds.

7. Isolation of the shoot apex by shallow incisions did not result in restriction of growth or the formation of buds outside the incisions.

8. No leaf-traces or leaf-gaps were formed in association with those primordia which had disappeared as a result of the experimental treatment.

9. The formation of abundant wound tissue in response to the cuts suggested that this might account for the dispersal of the primordia, by competing for substances necessary for the growth of primordia.

10. The hypothesis relating to the importance of the incipient vascular tissue in morphogenesis is supported by the experimental results. It is suggested that the control which the apex and existing primordia exert upon the growth and development of new primordia is probably a complex combination of hormonal relationships and requirements for nutrients and other substances.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. An apex in which P_1 was isolated by four shallow incisions. P_1 is still visible on the isolated plug (top of photograph), but has not grown since isolation. (Cf. Text-fig. 8.) Twenty-three days after isolation of P_1. (×40.)

Fig. 2. The same apex a fortnight later. P_1 is no longer visible on the isolated plug, and scales are present at its position. (Cf. Text-fig. 9.) Thirty-seven days after isolation of P_1. (×40.)

PLATE II

Fig. 3. Longitudinal section of an apex in which P_1 was isolated by a shallow adaxial incision immediately prior to fixing. The contents of the severed cells have disappeared. It will be seen that the cut affects only the superficial layer of cells. (×150.)

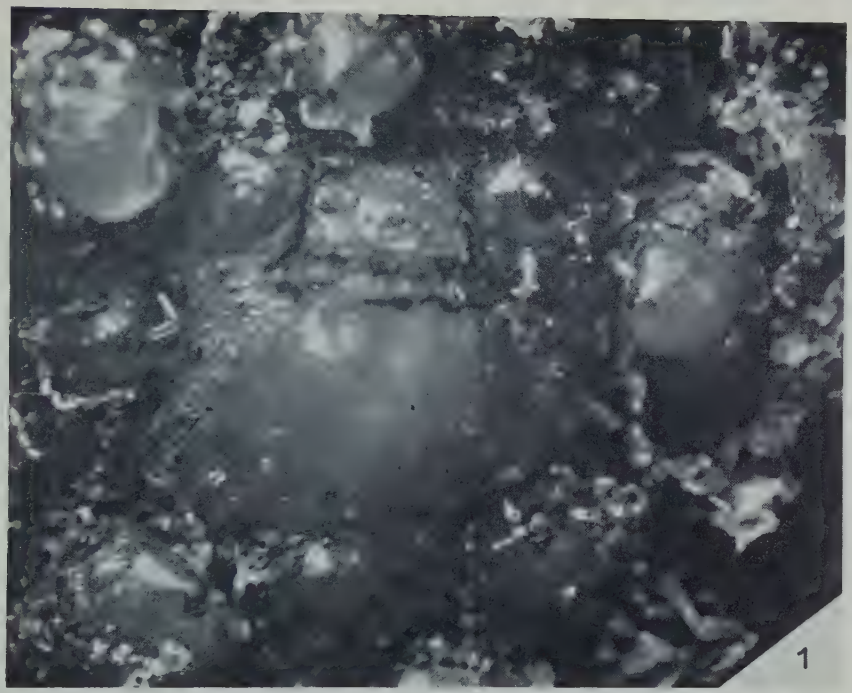
Fig. 4. Longitudinal section of an apex in which a young leaf primordium was isolated by a shallow adaxial incision one and a half hours before fixing. The contents of the severed cells have disappeared and there is some discoloration. The orientation of the leaf primordium with respect to the shoot apex is as in Fig. 3. (×150.)

Fig. 5. Longitudinal section of an apex in which a young leaf primordium was isolated by a shallow adaxial incision three days before fixing. The cells adjacent to the incision have become brown and suberized. Only the superficial layer of cells has been incised. The orientation of the leaf primordium with respect to the shoot apex is as in Fig. 3. (×150.)

Fig. 6. Longitudinal section of the specimen illustrated in Pl. I, Figs. 1 and 2, and Text-figs. 8–9. P_1, isolated by four shallow incisions, has disappeared, and there are no associated leaf-traces. Note the marked development of wound tissue beneath the adaxial cut; little wound tissue is associated with the abaxial cut. The shoot apex is to the left of the photograph, with P_9 on the right. Forty days after isolation of P_1. (×75.)

Fig. 7. Longitudinal section of a leaf primordium which was isolated closely by four shallow incisions after the apex had been allowed to grow on. The isolated P_1 primordium has developed as a normal, dorsiventral leaf primordium with conspicuous leaf-traces, notwithstanding the proximity of the incisions. (×75.)

Fig. 8. Longitudinal section of a leaf primordium isolated by four shallow incisions, which at first grew and developed as a normal leaf primordium, but eventually became parenchymatous. A leaf-trace is present in P_1, but does not extend into its parenchymatous tip. The shoot apex is to the right. (×75.)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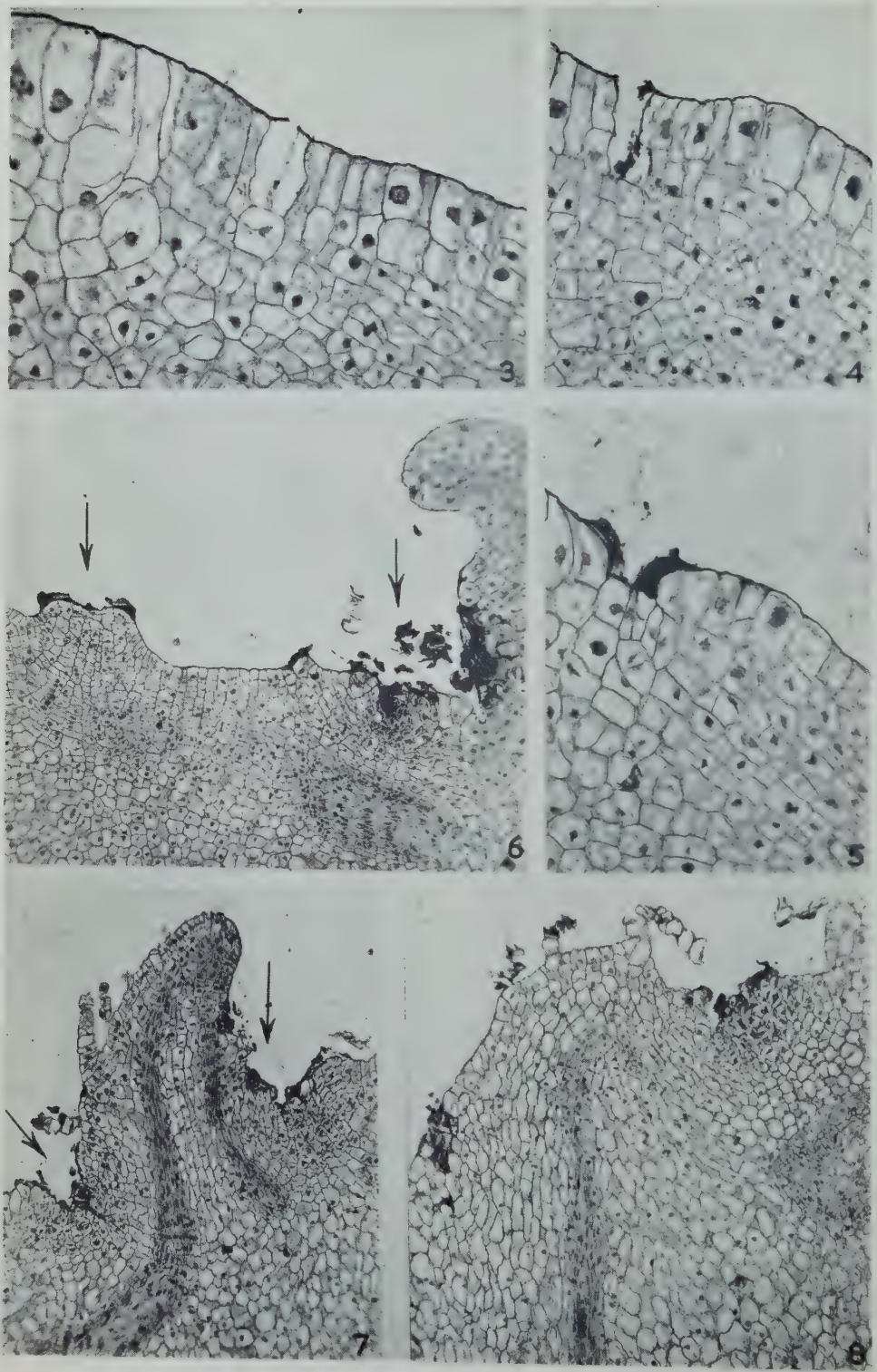
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Studies in the Comparative Physiology of Apple Rootstocks

I. The Effect of Nitrogen on the Growth and Assimilation of Malling Apple Rootstocks

BY

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With two Figures in the Text

ABSTRACT

The growth and net assimilation rates of four Malling apple rootstocks of varying vigour were observed under a wide range of cultural conditions, viz. soil, sand, and nutrient solution at three different hydrogen ion concentrations. The mean net assimilation rates of the stocks were found to be in order of vigour, irrespective of cultural medium, greater vigour always accompanying higher assimilation rate. There was a marked differential response to nitrogen, Crab C, a vigorous stock, being more efficient than M. IX at low nitrogen supply, whereas at higher levels the relative differences were less pronounced. Nitrogen deficiency also reduced very markedly the net assimilation rate of both stocks.

INTRODUCTION

MUCH research has been devoted to the study of the reciprocal relations of stock and scion and this has been reviewed in detail by Roberts (1949), but the problem still remains obscure. Little attention seems to have been paid to the more fundamental physiological characteristics of the unworked stocks, and there appears as yet to be no known physiological property with which the behaviour of the stocks can be correlated. Knight (1925, 1927) found slight differences in transpiration rate and water conduction of different stocks, but these were in no way related to vigour. There are few data available relating to carbon assimilation, nutrient uptake, or translocation rate of the stocks when grown under controlled conditions, although it is known that vigour or dwarfingness of a stock is greatly influenced by soil conditions (Hatton, 1926). It seemed necessary therefore, before considering the possible interaction of scion and stock, to examine the physiology of the unworked stocks without introducing any complexity due to the presence of a scion. Accordingly it was decided to compare the growth of a vigorous (Crab C) and a dwarfing rootstock (M. IX) at different nitrogen levels over a two-year period. Collateral evidence on the growth and assimilation rates of stocks of varying vigour (M. XVI, M. II, and M. IX) was also obtained in sand, soil, and nutrient solution cultures during the second year of the experiment.

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Materials and Methods

Crab C and M. IX rootstocks were grown in sand culture at the following nitrogen levels, 5, 17, 58, and 200 p.p.m. The nutrient solution of Batjer and Degman (1940) was employed with the modification that the total kation concentration was equalized by the addition of the requisite amounts of sodium sulphate. In consequence the sodium varied in the various treatments, being highest in the series with low nitrogen. Twelve replicates of each stock were used at each nitrogen level. Single plants were grown in sand culture in 10-inch earthenware pots coated internally with bitumastic paint to minimize water loss. At the time of planting (March 1953) all the stocks were weighed and a representative sample of twelve plants of each stock was taken for the determination of dry weight, and the regression of dry weight on fresh weight was used for estimating the initial dry weight of each experimental plant. Measurements of extension growth, leaf number, and leaf dimensions were made regularly. The leaf measurements, width and length of each leaf, were taken at monthly intervals. From the products of the leaf dimensions the leaf area was determined in the following way:

A representative sample of leaves from each stock was taken from the shoots soon after the beginning of growth, and in most cases at the conclusion of the experiment, and the absolute areas of the leaves determined by printing on ferrocyanide paper, cutting out, and weighing. These values were compared with the 'leaf products' and regression equations calculated. It was found that there was no significant difference between the regression equation at the beginning and end of the season under the different cultural conditions for each stock, and a mean regression line was therefore used for estimating the leaf areas of the two stocks throughout the season. The regression equations for the two stocks were:

$$\text{M. IX} \quad y = 0{\cdot}3 + 0{\cdot}66x \qquad \text{Crab C} \quad y = 0{\cdot}7 + 0{\cdot}64x$$

where y represents area in sq. cm. and x represents products of maximum length and breadth of leaves in cm.

At the end of the first season the plants grown at 200 p.p.m. nitrogen were harvested, and final dry weight of the various parts determined. This sample was taken in October before leaf-fall began.

The plants of the other treatments were repotted in winter into 12–inch pots similarly treated, and grown throughout the following season. In September 1954 the experiment ended and the final dry weights were taken.

From the total increment in dry weight and the mean leaf area the net assimilation rates of the stocks were calculated.

Experimental observations

Time of bud-break of the stocks was not influenced by treatment, the M. IX in both years coming into leaf earlier than the Crab C. Nutrients were at first applied daily and the sand leached with water once a week, but later in the season, owing to the appearance of scorch symptoms on some of the leaves,

feeding was reduced to twice a week. Within 2 weeks of the start of the treatments the leaves of M. IX and Crab C stocks receiving 200 p.p.m. nitrogen were darker green than those at the lowest nitrogen level (5 p.p.m.), and somewhat darker than those at the intermediate levels (58 and 17 p.p.m.) which were not distinguishable.

In mid-July 1953 brown purple areas and slight marginal scorch were present on some of the basal leaves of M. IX. There was only very slight scorch on the Crab C leaves. Symptoms were considered to be typical of magnesium deficiency and on two occasions a magnesium sulphate solution (60 p.p.m. Mg.) was given alone, but there was no noticeable beneficial effect.

TABLE I

Percentage of Leaves Showing Scorch (October 1953)

	Crab C				M. IX			
Nitrogen level	200	58	17	5 p.p.m.	200	58	17	5 p.p.m.
	9	11	13	22	16	21	37	40

The number of leaves showing any trace of scorch, however slight, was estimated for each stock in each treatment on October 8, and the percentage number of leaves showing scorch is given in Table I.

It will be observed that the percentage of scorch decreases with increasing nitrogen in both stocks and that M. IX shows more scorch than Crab C at all nitrogen levels. An analysis of co-variance, however, showed no significant relationship between leaf scorch and net assimilation rate of each stock.

TABLE II

Initial and Final D.W. and Increment in D.W. (g) of Stocks

		Crab C			M. IX		
Nitrogen level	Duration of expt.	Initial D.W.	Final D.W.	Increment D.W.	Initial D.W.	Final D.W.	Increment D.W.
5 p.p.m.	2 years	4·10	17·55	13·45 ± 1·08	3·27	11·10	7·83 ± 0·81
17 ,,	2 years	3·42	29·79	26·37 ± 3·01	3·36	23·58	20·22 ± 1·67
58 ,,	2 years	3·52	63·32	59·80 ± 5·50	3·31	54·20	50·89 ± 4·06
200 ,,	1 year	4·03	49·66	45·63 ± 3·88	3·68	44·23	40·55 ± 3·04

(Twelve replicates in each sample.)

In September 1954 the stocks grown at the three lower nitrogen levels were sampled. At this time many of the leaves showed signs of senescence, but no differential relationship either to stock or nitrogen level was apparent.

The effects of senescence on the assimilation rates of the stocks will be considered later.

EXPERIMENTAL RESULTS

Dry weight of the stocks

The mean initial dry weight (*g*) of the plants as calculated from the regression lines in the four nitrogen treatments, and the final weights as determined experimentally for the 2 years, with the increment in dry weight over the period,

TEXT-FIG. 1 *a*.

TEXT-FIG. 1 *b*.

Root-stocks M. IX (above) and Crab C (below) grown at four levels of nitrogen. (1) 200 p.p.m., (2) 58 p.p.m., (3) 17 p.p.m., (4) 5 p.p.m. Photograph taken in September 1953 at end of the first year's growth.

are given in Table II. It is apparent that the dry weight increment in Crab C at all levels of nitrogen was higher than in M. IX, but the differences between the varieties remained almost constant. In consequence, if vigour is assessed as relative size of plants, the relative vigour had been greatly affected by nitrogen level.

TABLE III

Percentage Distribution of Increment of Dry Matter

Nitrogen level	Crab C			M. IX		
	Leaves	Stems	Roots	Leaves	Stems	Roots
5 p.p.m.	26·4	38·6	35·0	32·1	33·5	34·3
17 ,,	28·0	43·4	28·5	31·1	42·4	26·5
58 ,,	29·9	51·3	18·8	30·8	49·9	19·3
200 ,,	22·8	52·3	24·9	23·6	50·1	26·3

Taking Crab C as 100, the values for M. IX with decreasing nitrogen level are respectively 89, 85, 76, 58. Judged by size, therefore, Crab C could be accounted as much more vigorous than M. IX at low nitrogen level, but at high nitrogen level the stocks would be regarded as almost equally vigorous. This is well seen in Text-figs. 1*a* and *b*.

Translocation

The percentage distribution of the increment in dry weight to the leaves, stems and roots during the experimental period is given in Table III.

TABLE IV

Increment in Dry Matter of Roots and Stems, Leaf Increment taken as Unity

Nitrogen level	Crab C	M. IX
5 p.p.m.	2·78	2·11
17 ,,	2·56	2·21
58 ,,	2·35	2·24
200 ,,	3·39	3·23

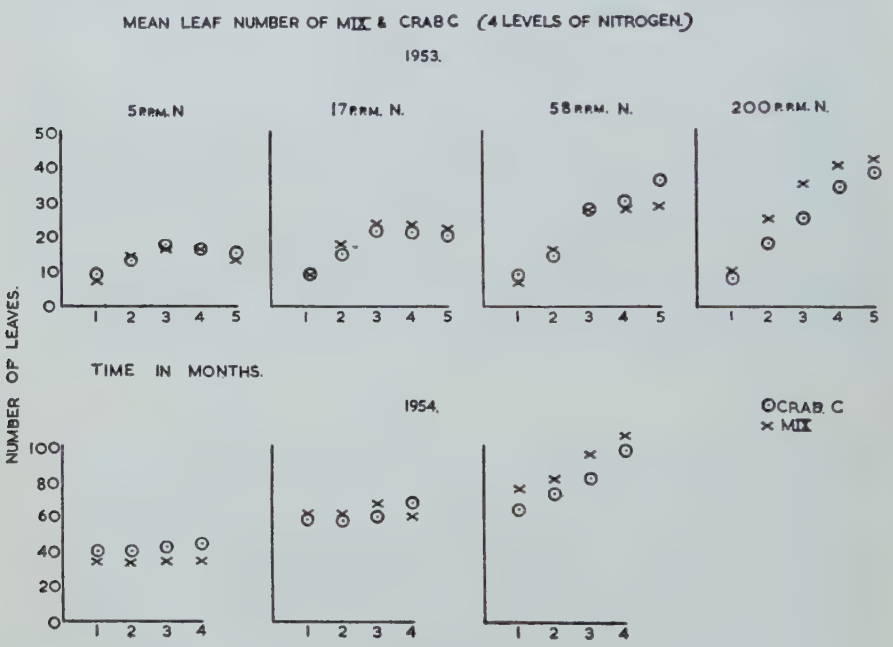
The distribution of assimilated dry matter among the plant organs in the two stocks shows the differential effect of nitrogen level. The relative amounts of leaf formed remain comparatively constant, but the relative sizes of stem and roots differ greatly with the nitrogen level. Between 58 and 5 p.p.m. the relative amount of growth made by the roots over the period of the experiment is doubled. Taking the increment of leaves as unity the relative values of the increment of stem and root are shown in Table IV.

The values for Crab C are uniformly higher than those of M. IX, and the differences between stocks become increasingly greater with increasing nitrogen deficiency. Since the increment in organs other than the leaves is almost entirely dependent upon material translocated from the leaves it

suggests that in the vigorous stock translocation is more effective. This difference in translocation was significant at the 0·05 per cent. level, but was found to be due entirely to the difference between the stocks at the lowest nitrogen level. With increasing nitrogen supply the effect tends to disappear.

Leaf growth of the stocks

The monthly measurements of leaves provided data on the growth of the leaf surface as related to stock and nitrogen level. The rate of leaf production was also observed. The mean leaf numbers of the two stocks at the different nitrogen levels are shown in Text-fig. 2.



TEXT-FIG. 2.

Certain differences between the stocks are apparent, namely, that at the highest nitrogen level in 1953, M. IX has significantly more leaves than Crab C, while at 5 p.p.m. this difference no longer exists. In 1954 at the highest nitrogen level (58 p.p.m.), M. IX again has in every case more leaves, at 17 p.p.m. nitrogen there is no significant difference, while at 5 p.p.m. nitrogen the order of the difference is reversed, M. IX having fewer leaves.

In 1953, at 200 and 58 p.p.m. nitrogen the leaf number increases throughout the season. At 17 p.p.m. maximum leaf number has been attained after 3 months, and 5 p.p.m. after 2 months. In 1954 the differences are still more marked and at the lower nitrogen levels no increase occurred after the first month. In comparing the data for the two seasons it will be apparent that the total number of leaves at all nitrogen levels is much higher in 1954. This is due to the fact that in the second season, not only the leaves on the main stem

but those on the axillary shoots are included, and in 1953 very little axillary growth took place. The relevant data are entered in Table V, which shows the number of leaves on main and lateral shoots and the average leaf size in 1954. Several points emerge: (1) Average leaf size is related to nitrogen level as expected; (2) leaf development on the laterals is almost complete after 1

TABLE V

Leaf Number in Main Axis, Lateral Axes, and Mean Leaf Size

		Leaf No.: main axis				Leaf No.: laterals				Mean leaf size, cm.[2]			
Treatment	Stock	June	July	Aug.	Sept.	June	July	Aug.	Sept.	June	July	Aug.	Sept.
Nitrogen level 5 p.p.m.	M. IX	8·7	10·0	9·7	18·5	25·6	24·3	24·8	17·0	3·8	4·5	5·0	5·9
	Crab C	12·3	13·3	13·5	14·6	28·8	27·8	29·3	29·3	4·3	5·2	5·6	5·9
17 ,,	M. IX	8·2	11·8	16·3	25·3	52·6	48·5	53·3	34·2	3·7	4·9	5·3	6·6
	Crab C	11·4	13·9	15·3	24·1	46·7	42·8	44·7	44·7	4·5	4·6	4·7	6·8
58 ,,	M. IX	12·4	16·8	22·0	30·8	66·1	68·0	75·8	76·7	4·1	5·5	6·0	8·1
	Crab C	15·2	22·0	24·0	34·2	51·1	51·7	60·4	64·6	3·4	5·0	5·7	10·3

month, whereas on the main axis continued leaf growth goes on; (3) if the number of lateral leaves is a measure of apical dominance, then it is evident that apical dominance is increased by nitrogen deficiency; (4) at all nitrogen levels the average leaf size increases throughout the season and a large differential effect of nitrogen is seen.

Leaf areas

The mean leaf areas of the plants for the whole season in each treatment calculated from the regression lines as described previously are given in Table VI together with the relative values of Crab C (M. IX taken as 100).

TABLE VI

Mean Leaf Area for Whole Season (sq. cm.). In Brackets, Relative Values, corresponding M. IX taken as (100)

	Crab C		M. IX	
Nitrogen level	1st year	2nd year	1st year	2nd year
5 p.p.m.	118 (117)	198 (142)	102 (100)	140
17 ,,	189 (95)	297 (102)	199	291
58 ,,	349 (89)	506 (90)	393	562
200 ,,	456 (83)	—	551	—

As the level of nitrogen is raised the mean leaf area of Crab C which at 5 p.p.m. is greater than that of M. IX falls considerably below at 58 p.p.m.

The mean leaf area of Crab C expressed as a percentage of that of M. IX at each sampling time throughout the season is given in Table VII.

At the first sampling in 1953 M. IX has approximately twice the leaf area of Crab C at the highest nitrogen level. At intermediate levels the difference is reduced, and at 5 p.p.m. nitrogen the leaf areas are approximately the same, while in 1954 Crab C has a considerably greater leaf area than M. IX at June 1 at this nitrogen level. This is of interest when considering time of bud burst,

which is somewhat later in Crab C. Thus on May 12, 1953, only 30 per cent. of Crab C and 100 per cent. of M. IX stocks had shown bud burst. It appears therefore that the earlier bud break does not result in more rapid leaf growth at the lowest nitrogen level, but at the higher nitrogen levels the early differences are maintained.

TABLE VII

Mean Leaf Areas of Crab C throughout the Season (M. IX as 100)

Nitrogen level	1953					1954			
	June	July	Aug.	Sept.	Oct.	June	July	Aug.	Sept.
5 p.p.m.	98	92	116	120	143	149	155	148	115
17 ,,	85	65	98	102	103	106	109	94	104
58 ,,	65	59	85	92	111	78	85	84	117
200 ,,	57	60	82	83	96	—	—	—	—

Net assimilation rate

The mean leaf area of each plant was determined by integration of the leaf area curves over the experimental period, and net assimilation rate (N.A.R.) was measured as the increase in dry weight of the whole plant, calculated as g. per sq. dm. of leaf surface per week. The data for the highest nitrogen

TABLE VIII

Net Assimilation Rates (g. D.W./per sq. dm./week)

Treatment	Time	M. XVI	M. II	M. IX	Crab C
Sand culture					
N 200 p.p.m.	June 3–Sept. 30, 1953	—	—	0·379±0·0062 (n = 12)	0·511±0·0074 (n = 12)
N 58 p.p.m.	June 3–Sept. 30, 1953 +June 1–Sept. 1, 1954	—	—	0·160±0·0046 (n = 12)	0·210±0·0044 (n = 12)
N 17 p.p.m.	,,	—	—	0·123±0·0027 (n = 12)	0·164±0·0041 (n = 12)
N 5 p.p.m.	,,	—	—	0·094±0·0037 (n = 11)	0·129±0·0041 (n = 11)
Soil culture	June 1–Sept. 1, 1954	0·686±0·015 (n = 6)	0·627±0·016 (n = 6)	0·494±0·032 (n = 5)	—
Sand culture	,,	0·767±0·034 (n = 6)	0·690±0·032 (n = 6)	0·511±0·016 (n = 6)	—
Water culture					
pH 3·6 aerated	,,	0·447±0·014 (n = 6)	0·351±0·029 (n = 7)	—	—
pH 4·0 ,,	,,	0·464±0·026 (n = 11)	0·359±0·025 (n = 6)	0·296±0·007 (n = 2)	—
pH 5·5 ,,	,,	0·629±0·043 (n = 8)	—	—	—

(n = number of replicates.)

level were determined at the end of one year's assimilation period, while those for the other nitrogen levels were determined at the end of the second assimilation period, the leaf weights from the first period being added to those of the final sampling.

It will be seen (Table VIII) that the vigorous stock Crab C has without exception a higher mean N.A.R. than M.IX, and this is irrespective of nitrogen level. Nitrogen deficiency has very markedly reduced the net assimilation rate.

Subsidiary experiments

The relevant data from a number of experiments carried out in 1954 are given below. In these experiments M. IX, M. II, and M. XVI were grown in (*a*) sand culture, (*b*) John Innes potting compost, (*c*) in nutrient solution at three hydrogen ion concentrations. In this way, the growth of the three stocks was compared under widely different cultural conditions.

Routine observations on growth were made as before during one summer. The results of net assimilation rates are of interest, and are shown in Table VIII.

It will be observed that the three stocks of varying vigour show a marked gradation of N.A.R., the vigorous stocks always being higher. The actual values for soil and sand cultures are not significantly different. The plants in water culture have a lower N.A.R. and this rises with pH. In stock M. XVI at pH 5·5 the N.A.R. is almost as high as that of sand culture.

No differential effect of stock on amount of translocation from the leaves to other portions was found, and accordingly the data are omitted.

The most striking conclusion taking the results of these experiments is that in all stocks under these conditions the N.A.R. is in order of vigour, and that greater vigour accompanies higher assimilation rate.

Discussion

It has been shown that the levels of nitrogen used have significantly affected the growth of rootstocks. As these results are in agreement with those of Batjer and Degman (1940), who investigated a wide range of nitrogen levels on the growth of one-year-old York Imperial apple trees, it is not necessary to discuss this aspect of the work further. The aim of the experiment described was to investigate the differential effects of the levels of nitrogen on the growth of a vigorous and dwarfing rootstock, field observations having shown that the vigour of trees grafted on various rootstocks is liable to vary under different soil conditions. The results demonstrate that there has been a marked differential behaviour of the two stocks, the growth of Crab C being significantly greater than M. IX irrespective of nitrogen level. In assessing this effect the measure of vigour is of importance, as the differences may be stated either relatively or in absolute terms. On a relative basis the difference between the stocks ranges from 73 per cent. at the lowest nitrogen level to 12 per cent. in favour of Crab C at the 200 p.p.m. level, whereas on an absolute basis the difference in total weight is almost constant (Table II). This difference in response to low nitrogen is also well reflected in the mean leaf area of the stocks, that of Crab C being considerably greater at 5 p.p.m. nitrogen and falling below that of M. IX at 58 p.p.m. With limited nitrogen supply therefore Crab C is the more efficient plant. That this effect is due neither to a higher nitrogen content of the leaves nor to higher content of other major elements is shown by analyses carried out in October 1953, and these values for the two stocks are given below in Table IX.

This higher efficiency of Crab C is not of necessity related to vigour, and further analysis of this effect awaits completion of chemical analyses of the whole plant. However, this finding confirms the so-called 'soil effect' on vigour as noted by Hatton (1926) at least in regard to one factor—nitrogen supply.

The data presented also show that nitrogen level has had a marked effect on mean assimilation rate, the plants at the high nitrogen level (58 p.p.m.) having approximately twice the rate of those at the lowest level (5 p.p.m.). There appear to be a number of possibilities in explaining this effect, either that increased nitrogen *per se* has increased the efficiency of the assimilation process or has stimulated the production of young active leaves or that both

TABLE IX

Concentration (g) per cent. in Dry Leaves

Conc. of N in nutrient (p.p.m.)	N.	P.	Mg.	Ca.	K.
M. IX					
200	2·44±0·10	0·34±0·02	0·212±0·024	1·16±0·075	1·7±0·07
58	2·13*±0·08	0·46±0·02	0·173±0·017	1·19±0·032	2·2±0·14
17	1·91±0·04	0·70±0·07	0·157±0·058	1·31±0·082	2·3±0·15
5	1·81±0·07	0·78±0·07	0·123±0·023	1·32±0·036	2·5±0·07
Mean	2·07	0·57	0·166	1·23	2·2
Crab C					
200	2·41±0·05	0·25±0·02	0·195±0·028	0·83±0·03	1·6±0·04
58	2·03±0·16	0·45±0·07	0·128±0·019	0·80±0·03	2·0±0·16
17	1·90±0·10	0·69±0·08	0·096±0·016	0·83±0·03	2·4±0·22
5	1·67±0·07	0·64±0·06	0·074±0·013	0·80±0·04	2·8±0·25
Mean	2·00	0·51	0·123	0·82	2·2

(Five replicates in each case except *, where n = 4.)

causes are operative. There is some evidence for a direct effect of nitrogen on assimilation rate: of wheat and rye (Gassner and Goeze, 1934); of *Sinapis alba* (Müller and Larsen, 1935); and of wheat, barley and mangolds (Watson, 1947). In the apple Batjer and Degman (1940) measured the photosynthetic rates of detached leaves from one-year-old trees grown under a wide range of nitrogen concentrations. They claimed that the apparent rate of photosynthesis per unit area was roughly proportional to the amount of nitrogen supplied, and that the assimilation rate at 60 p.p.m. nitrogen was approximately twice that at 5 p.p.m., which is very close agreement with results obtained in the present investigation for these levels by an entirely different method. At the highest nitrogen levels (168 and 60 p.p.m.) Batjer and Degman found no difference in assimilation rate of detached leaves. There are, however, no data available concerning the 'condition' of those leaves used in obtaining their results at the different nitrogen levels as regards deficiency symptoms, or senescence, &c. On the other hand, other investigators have found no direct effect of nitrogen on assimilation rate. Thus Gregory (1926), Gregory and Richards

(1929), Crowther (1934), and Heath and Gregory (1938) using direct carbon dioxide measurements and methods of growth analysis claimed that nitrogen treatment had no effect on N.A.R. up to flowering time. In the experiment described by Gregory (1926), in which barley was grown under a wide range of nitrogen levels, the net assimilation rates were calculated covering stages up to maximum leaf areas, i.e. before leaf senescence began. In the apple stocks experiment it will be observed that at the lowest nitrogen level there is no increase or at least very little increase in leaf area or leaf number throughout the whole season; the leaves formed at the beginning of the season are thus the only ones that function in photosynthesis and these leaves showed typical symptoms of nitrogen deficiency and senescence. At the higher nitrogen levels leaf formation continued much later in the season. Accordingly it is possible that any effect on assimilation rate may be attributed to the ageing of the leaves and the absence of young leaves rather than to the direct effect of nitrogen.

Heath and Gregory (1938) have emphasized the comparatively small range of N.A.R. over a wide range of species and conditions; but that the N.A.R. is not a constant for different plants (wheat, barley, mangolds) has been shown by Watson (1947). Similarly with perennial species, on which only limited growth analysis data are available, Monselise (1953) found a significant difference in N.A.R. between sour orange and other citrus rootstocks. The present work has shown that there is a highly significant difference in N.A.R. of the different apple rootstocks, and irrespective of a wide range of cultural conditions (soil, sand and water) the vigorous stocks have a greater N.A.R. than the less vigorous ones. It is not possible as yet to analyse further the differences found in assimilation rates; for this, frequent samples taken at short intervals will be necessary. In this way the different characteristics of the stocks, e.g. time of bud break, leaf senescence and the interaction of these properties with climatic conditions could be taken into account. The fact that has been established from these experiments, although on an empirical basis, is that higher assimilation rate accompanies greater vigour. It should perhaps be emphasized that results obtained during the first 2 years of growth may not be applicable to later behaviour of the plants, but from a practical point of view it appears possible that these findings may be of value in forecasting the vigour or otherwise of new rootstocks.

Summary

1. The effect of nitrogen on the growth and net assimilation rate of a dwarfing (M. IX) and a vigorous (Crab C) apple rootstock in sand culture was studied. Growth analysis observations were made on these stocks over a two-year period, and on stocks M. IX, M. II and M. XVI grown in soil, sand, and water cultures at different pH levels over a period of 1 year. In this manner the growth of the stocks was compared over a wide range of cultural conditions.

2. Nitrogen level was found to have a highly significant effect on growth. There was a marked differential response to nitrogen. On a relative basis Crab C exceeded M. IX by 73 per cent. at 5 p.p.m. nitrogen, whereas at 200 p.p.m. nitrogen the relative difference was 12 per cent. On an absolute basis the difference in weight was almost constant. The greater efficiency of Crab C was not due to a higher nitrogen, phosphorus, or potassium content of the leaves, the concentration being identical in the two stocks.

3. Nitrogen deficiency reduced very markedly the N.A.R. of both stocks, and the bearing of this on the relation between N.A.R. and nitrogen is discussed.

4. The net assimilation rates of all the stocks grown under a wide range of cultural conditions are in order of vigour of the stocks, greater vigour always accompanying higher assimilation rate. Statistically these results are highly significant.

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Studies in the Genus *Elaphoglossum*

IV. The Morphological Series in the Genus and their Phylogenetic Interpretation

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PART II[1]

THE INTERPRETATION OF THE RESULTS OF THE COMPARATIVE EXAMINATION

The representativeness of the material examined. In the following sections the collections from the New World are considered separately from those of the Old. It is generally agreed that the floras of the tropics of the eastern and western hemispheres have been separate since the beginning of the Tertiary

[1] Part I of this paper appeared in *Annals of Botany*, N.S. xix. 173–99.

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period (Chaney, 1940) and it is conceivable that the evolution in the genus in the two areas may have proceeded independently since that time. Since the largest collection has come from the American continent, this is analysed in detail and what is known of the species of the Old World is incorporated in the interpretation of the results.

The American collection falls into two groups, the Jamaican and the Ecuadorean. As stated earlier, all except two of the Jamaican species have been investigated. Only one Jamaican species is reported as endemic, namely *E. nematorhizon* (Morton, 1954) and this may yet be found elsewhere in the West Indies or Central America, the *Elaphoglossa* of many parts of which are still little known. Twenty of the twenty-seven Jamaican species are also known from the American mainland. A strong resemblance between the *Elaphoglossa* of Central America and Jamaica is to be expected, since Jamaica, Haiti and Puerto Rico formed a continuous ridge of land connected with Honduras and Nicaragua as recently as the Miocene (Schuchert, 1935). In addition, fern spores may be blown continuously from the mountains of Central America to those of the West Indies. Although the surface winds of the Caribbean are predominantly north-easterly, above 20,000 ft. they are predominantly westerly (Meteorological Office, 1954). No investigations into the aerial dispersal of fern spores in tropical regions have yet been made, but Polunin (1951) has found them frequently at 5,000 ft. and occasionally up to 5,350 ft. over northern Canada. In addition, pollen grains have been detected up to 10,000 ft. north of Alaska (Polunin, 1954). Over Panama even the hairy spikelets of the grasses *Paspalum urvillei* and *Andropogon bicornis* have been caught in aeroplane traps at 4,000 ft. (Chase, 1954). In view of these facts it does not seem improbable that in Central America generally, where a great number of fern spores are continually being discharged, a significant proportion might be carried up to heights of 20,000 ft. or more, especially over the mountains, and dispersed in the upper air currents. In descending over the Caribbean or Atlantic they would be caught in the north-easterly Trades and some deposited on the windward sides of the islands, of which Jamaica occupies a central position. The richest fern flora in Jamaica is found in the gullies on the windward sides of the mountains (Shreve, 1914) and this may be due, not only to the greater precipitation on this side, but also to immigrant spores.

For these reasons the Jamaican *Elaphoglossa* can be regarded as a sample of the genus as it is represented in the Caribbean area. The presence of one possible endemic does not affect this view, since only the characters α, β, and γ are to be considered and in respect of these the possible endemic is in no way peculiar. Moreover, all the sections of the genus recognized by Christ (1900), with the exception of the small sections *Gymnoglossa* and *Undulata*, are represented in Jamaica.

The Ecuadorean collection can similarly be regarded as a sample of the species of *Elaphoglossum* growing in the Equatorial region of this country. The expeditions made in Ecuador together constituted a number of transects

from high alpine to low mountain rain-forest conditions. All the specimens of *Elaphoglossum* seen on these expeditions, except those which had certainly been encountered earlier in the same locality, were collected.

A large proportion of the species recorded from tropical Africa and from Malaya have been examined and a few from elsewhere.

The phylogenetic significance of the morphological series. That variation in a character in a genus shows a connectedness is inevitable, since a mutant must show a relatedness to that from which it springs, but the confining of the variation in the characters α, β, and γ to an almost linear series is very striking. It cannot be an illusion engendered by considering only the genus *Elaphoglossum*, since non-linear variation in any of these characters would not cause the species displaying it to be placed in any other genus. Nor does it appear likely from an examination of numerous herbarium specimens from all parts of the tropics and sub-tropics that any other states of these characters are to be found. Variation of this type indicates the basic genetic similarity of the material, but it need not imply orthogenesis, in the sense in which this term is used by Huxley (1942), meaning an evolutionary trend caused by some inner momentum. This point is discussed further after evidence for the phylogenetic order of the series has been presented.

The genus has no known fossil history. When it is compared with the rest of the polypodiaceous ferns, the evidence points to its being of comparatively recent origin (Bower, 1928). The lack of a well-established fossil history of any polypodiaceous ferns until Tertiary times suggests that they did not arise until the late Jurassic or Cretaceous period. Their rise, therefore, would have been contemporaneous with, or a little later than, that of the Angiosperms. *Elaphoglossum* is intimately associated with the moist forests of tropical mountains and many of its species are epiphytes. It is known that many of the arborescent angiospermous genera of these forests were already in existence at the beginning of the Tertiary period, often associated in floras, such as the Wilcox flora of the south-eastern United States (Berry, 1916) and the London Clay flora (Reid and Chandler, 1933), which indicate a wide extension at that time of tropical and sub-tropical conditions. From this and other evidence (Berry, 1918; Chaney, 1947), it follows that since the beginning of the Tertiary and possibly since the beginning of the Cretaceous period (Axelrod, 1952) there has been continuously in existence, although less widely distributed now than formerly, the environment to which *Elaphoglossum* today is almost wholly confined. Even during the Ice Age, there was probably little change in conditions in the Equatorial belt except at high altitudes (Brooks, 1926).

If the view is correct that *Elaphoglossum* is a fern of recent, possibly late Mesozoic, origin, then the differentiation of its many species may well have progressed in the widespread tropical angiospermous forests of the Tertiary and recent period. In view of the ease with which the bark of angiospermous trees is colonized by mosses and liverworts in moist tropical conditions, there would be every opportunity for spores of *Elaphoglossum* and other ferns to be

caught and to germinate on stems and branches and for the epiphytic habit to become established and eventually obligate in certain species. It further follows from the prolonged existence of the environment provided by these forests that, if the evolution of *Elaphoglossum* has been confined to them, the genus has never had to overcome violent changes of climate or habitat. There would then be every reason to expect all viable variation, except that eliminated by accident or catastrophe, to be continued within the genus and to be reflected in the range of form it displays. Moreover, there would be no reason to expect the disappearance of the initial states of those characters showing serial variation and which, in combination with other characters, were not subject to natural selection. It is argued in a following section that there is no evidence to suggest that the various combinations of the different grades of the characters α, β, and γ are subject to selection. One grade of each character may, therefore, be expected to represent the state of that character, in the earliest species of *Elaphoglossum*. This does not imply that the ancestral species of the genus exist today unchanged. Natural selection has in all probability acted upon certain characters, so that a group of modern species may be close to the ancestral species in some characters, but not in others.

These general considerations lead to the conclusion that the morphological series are also phylogenetic series. Whether the initial state of a character lies at an extreme or within its morphological series remains to be decided in the light of other evidence.

Evidence for the antiquity of α_1. The extremes of the α series show marked differences in geographical distribution. The simple α_1 occurs throughout the tropics, but it is probably commoner in the Old World, especially in Malaysia, than in the New. It is significant that Holttum (1946), a systematist more familiar with the Asiatic species than the American, regards the possession of a creeping rhizome with two-ranked leaves, which would indicate the α_1 condition, as a character defining the genus, whereas in South America it occurs in only a minority of the species. On the other hand, α_4 appears to be confined to the New World and to be found there more frequently in those areas which have been colonized by plants since the beginning of the Tertiary period. Since *E. spathulatum* var. *pusillum* from Jamaica shows an α_4 stele, it was expected in *E. spathulatum* from Ceylon (*Beddome s.n.*) and Rhodesia (*Milne-Redhead 4334*), but both these have α_2 steles. It has since been discovered that *E. spathulatum* from Ecuador (*Camp E—3388*) also has an α_2 stele. *E. phanerophlebium* from Madagascar (*Perrier de la Bathie, 12039*) and *E. clarenceanum* from the island of San Tomé (*Quintas, 1379*), both of which have short ascendant rhizomes, have α_3 steles in which a clear dorsiventrality is retained throughout. The α_4 condition has not been suspected in any other species from the Old World. If it is not absent, it must be extremely rare.

In addition, the species whose steles show the α_1 condition are almost always unspecialized, with simple creeping rhizomes and fronds whose laminae are quite normally constructed. The α_4 condition, on the other hand, is confined to a few species with thin papery fronds, possessing hydathodes and bearing

complex bristle scales. Fronds possessing so little structural tissue would collapse if subject to any desiccation and the species showing them are confined to areas of almost continuous precipitation.

The general distribution of the α_1 condition and the fact that the species showing it are unspecialized are held to point to the primitiveness of this grade and the restricted distribution of α_4, and its confinement to a few highly specialized species to its recentness. The intermediate states of α show a general distribution, perhaps tending to be commoner in the New World than in the Old. The morphological series in α can now be interpreted phylogenetically and it is seen that in this instance the phylogenetic order is the same as the morphological order.

No such marked differences of distribution have been found in the grades of the characters β and γ, although it is clear that β_1 and γ_4 are very poorly represented in, or absent from, Malaysia. The recognition of the primitive states of β and γ can, however, be based upon other arguments.

The frequencies of representation of the different grades of the characters α, β and γ. From the data in Table V it is possible to study the frequency with which the different grades of the characters α, β, and γ occur in species of *Elaphoglossum*. The second grades of both α and β have the highest frequencies in both the Jamaican and Ecuadorean samples. In the Old World species the first grade of α and the second of β have the highest frequencies. In the Jamaican sample the second grade of γ is the commonest, in the Ecuadorean the third, and in the Old World the second, third, and fourth grades of γ are equally represented. The first does not appear at all in the Old World species examined.

The combinations of the characters taken two at a time. In order to test whether the characters α, β and γ are independent of each other, they are first considered two at a time, since the relationship of two variables is more easily elucidated in the early stages of analysis than that of three taken simultaneously.

(i) *The combinations of α with β.* It will be seen (Fig. 9 (i) and (iv)) that the combinations $\alpha_1\beta_3$ and $\alpha_2\beta_2$ occur more frequently than any others. The numbers in the third and fourth grades of α are too small to permit a direct test for independence; they are consequently summed and a value of χ^2 is calculated for the 3 by 3 table obtained (Table VIII). In this calculation the discrepancy between the observed and the expected values in each cell is diminished by 0·5 to correct for the smallness of the numbers involved (Yates, 1934). For both the Jamaican and Ecuadorean samples the values of χ^2 correspond to probabilities so low that the grade of α cannot be considered independent of that of β. The greatest contributions to the values of χ^2 in both samples come from the cells $\alpha_1\beta^3$ and $\alpha_{3+4}\beta_1$ by virtue of their over-representation.

(ii) *The combinations of β with γ.* The frequencies of the different combinations are shown in Fig. 9 (ii) and (v). The value of χ^2 (Table VIII) for the Ecuadorean sample is such that the hypothesis of the independence of β and γ must

be discarded. The smaller Jamaican sample gives the same indication, although not so conclusively. The cell $\beta_3\gamma_2$ contains frequencies markedly greater than those expected in both samples. In the Jamaican sample the cell $\beta_1\gamma_4$ also gives a high contribution to χ^2 by virtue of its under-representation. It is to be observed also from these tables that γ_1 and γ_3 are combined with β in very much the same way. In the Ecuadorean sample, for example, γ_1 is found only with β_2 and in both samples γ_3 occurs more frequently with β_2 than with any other grade of β. The significance of this will be discussed later.

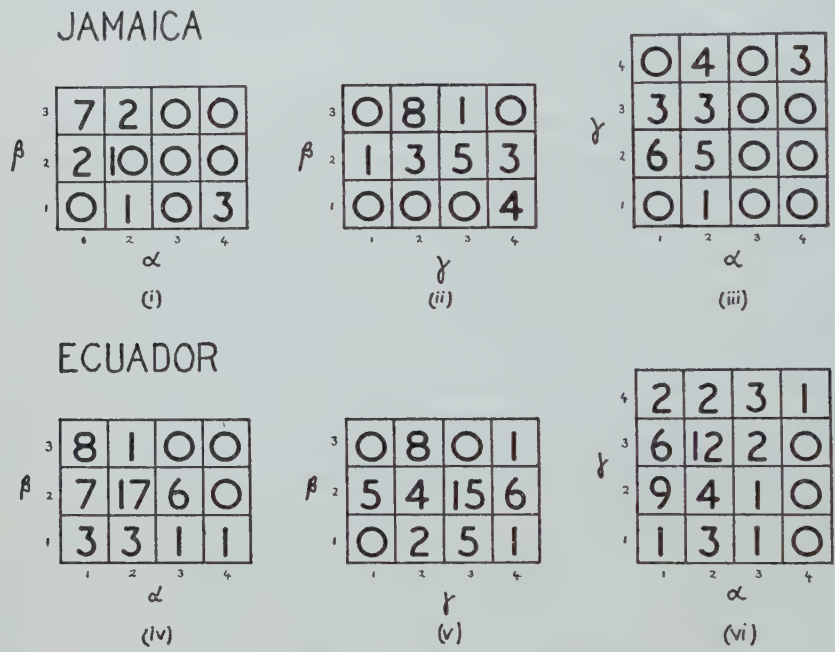

FIG. 9. The combinations of α, β, and γ considered two at a time.

(iii) *The combinations of α with γ.* Since a high proportion of the species graded α_1 are also β_1 and conversely a high proportion of those graded β_1 are also α_1, it follows, since a high proportion of those graded β_1 are also γ_2, that a high proportion of those graded α_1 are also γ_2. This is indeed found (Fig. 9 (iii) and (vi)), but the distribution of the frequencies is such that it might have occurred even where there was no reason to expect a lack of independence in the characters (Table VIII). Nevertheless, in the Ecuadorean sample considerable departures from expectation are given by the cells $\alpha_1\gamma_2$ and $\alpha_{3+4}\gamma_4$ and in the Jamaican by the cell $\alpha_{3+4}\gamma_4$, all of which contain frequencies greater than expected. It will be noticed, too, that in the Ecuadorean sample γ_1 and γ_3 are combined with α in the same way, both being more frequently combined with α_2 than with any other grade of α. In these and other respects the general pattern of distribution approaches that shown by the combinations of β and γ.

The association of certain grades of the characters α, β *and* γ. The grades of a combination which occurs more frequently than would be expected, assuming the characters to be independent, are said to be associated. It will have been observed that the results of the analyses of the Jamaican and Ecuadorean samples and the associations between the grades of α, β, and γ in each are very similar. This is all the more striking when it is realized that these two regions are some 1,200 miles apart and that the samples had only two species in common (*E. apodum* and *E. tectum*). Certain combinations, namely, $\alpha_3\beta_3$, $\alpha_4\beta_3$, $\alpha_4\beta_2$, $\alpha_4\gamma_1$, $\alpha_4\gamma_2$, $\alpha_4\gamma_3$, $\beta_1\gamma_1$, and $\beta_3\gamma_1$ have not been discovered in material from any source, nor have they been suspected in any herbarium specimens.

TABLE VIII

Tests for Independence of α, β *and* γ

Table	Degrees of Freedom	χ^2	
$\alpha_{1,2,3+4} \times \beta_{1,2,3}$			
Jamaica	4	16·98	$P < 0{\cdot}01$
Ecuador	4	9·79	$0{\cdot}02 < P < 0{\cdot}05$
$\gamma_{1,2,3,4} \times \beta_{1,2,3}$			
Jamaica	6	13·46	$0{\cdot}02 < P < 0{\cdot}05$
Ecuador	6	15·36	$0{\cdot}01 < P < 0{\cdot}02$
$\alpha_{1,2,3+4} \times \gamma_{1,2,3,4}$			
Jamaica	6	7·45	$0{\cdot}20 < P < 0{\cdot}30$
Ecuador	6	7·87	$0{\cdot}20 < P < 0{\cdot}30$

Although the criteria used to discriminate species include, explicitly or implicitly, the characters considered here, they are, nevertheless, independent of them, so that the grouping of the material into species cannot influence in any way the frequencies with which the different grades occur, nor automatically render any particular combination impossible. The associations are, therefore, the result of biological influences. They may be determined by genetic or metabolic forces within the plant or by factors in the external environment which eliminate or reduce the representation of certain combinations. Nothing has been observed in material from elsewhere to contradict the conclusion that the results obtained from the investigation of the American species are not accidental, but reflect real tendencies in the genus which are worthy of further investigation.

The possible physiological or genetical relationship between the characters. There is no *a priori* reason to expect a close ontogenetic or physiological connexion between the characters which have been described in the foregoing sections. Since they are being expressed in one plant they are necessarily related in a general way, but the characters are separated spatially and the development of any one is not directly consequent on that of any other. Nor is there any reason to expect that any state of one of the characters would automatically exclude any state of the other two. There is thus no apparent reason why variation in the three characters should not proceed independently.

Indeed, the fact that in the sporelings of some species the scales and the petioles, which are the same in the juvenile as in the mature plants, are combined with a succession of stelar forms as the rhizome comes to maturity is evidence of the lack of any obligate relationship between the state of the stele and that of the petiole and scales. It appears unlikely, therefore, that the associations between the grades of characters arise from a physiological or genetical dependence of one character upon another.

The possible selective advantage of the more frequent grades and combinations. Even were the characters varying independently, a preponderance of certain combinations of grades would occur if they possessed some selective advantage. Stebbins (1951) has put forward this explanation to account for the fact that some combinations of characters occur more frequently than others in dicotyledonous Angiosperms. While this may be a valid explanation of the situation in the Angiosperms, it is, nevertheless, clear that selective advantage is assumed throughout rather than demonstrated. The evidence for selective advantage of certain characters or combinations of characters is very difficult to obtain, since it calls for prolonged observations upon populations. It is possible, however, to examine the likelihood of selection acting upon the characters considered in *Elaphoglossum*. When the variations in the stele, petiole, and scales of the frond are considered from the point of view of the economy of the plant, it is very difficult to see why any of these variations or combinations of them should possess a selective advantage. In the Ecuadorean sample, for example, the combination $\alpha_2\beta_2$ has 17/21 of the total representation at the α_2 grade, while that of the combination $\alpha_2\beta_1$ is only 1/21. The figures for the Jamaican sample are as striking. It is difficult to see how a slightly less pronounced and definite joint in the petiole and a slight change in the associated parenchyma would confer an advantage of this order. Again, there is no evident reason why the β_2 condition should have a greater advantage at the α_2 grade than at the α_1, yet at the former it has a far greater proportional representation than at the latter in both samples. Similarly it is difficult to see why simple scales should be more biologically successful than complex when combined with simple steles and complex scales more successful than simple when combined with complex steles.

It is thus impossible to predict from a consideration of the characters themselves that any of their combinations will confer an advantage on the species displaying them. The possibility remains that it is some physiological state connected with them which is of importance. This physiological state may influence either the ecological requirements of the plant, so that certain combinations will be associated with certain habitats, or it may influence the general metabolic or reproductive processes of the plant, rendering them more or less efficient. If the species with the more frequent combinations do in fact possess an advantage of this latter kind, then it may be expected to result in the species themselves becoming more numerous than those with the less frequent combinations.

Nothing was seen in the field to support either of these possibilities. Al-

though no detailed studies have been made of the ecology of *Elaphoglossum*, it was noted in the examination of the Ecuadorean sample that all the combinations of α, β and γ present in the species of the páramo were also present in those of the lower mountain forest, the two extremes in the range of habitats in which the genus occurs. Although the combination $\alpha_4\beta_1\gamma_4$ was found in only four species in the two American samples, all of them, by virtue of the structure of their fronds, confined to continuously damp conditions, the structural features involved were not concerned essentially with the characters α, β and γ. There is no indication, therefore, that any of the combinations of the characters possesses an ecological significance.

With regard to the frequency of occurrence of individual species, some data are available from Ecuador. Since in a new area in this country specimens were taken of all the stands of *Elaphoglossum* encountered, even of those which closely resembled previous collections, lest they proved different on closer examination, the frequency of collection gives some measure of the representation of a species. It will be observed from Table V that of the seventeen species collected in Ecuador with the frequent $\alpha_2\beta_2$ combination, only seven were collected twice or more and the remaining ten were seen only once and in small quantity. *Bell 93*, on the other hand, with the much less frequent $\alpha_1\beta_2$ combination was ubiquitous on the páramo. *Bell 487* ($\alpha_2\beta_1$) and *Bell 503* ($\alpha_1\beta_2$) were other species with the less frequent combinations which were collected more than once and which occurred in quantity. A marked biological advantage cannot, therefore, be associated with the more frequent combinations of α, β and γ and the reasons for the success of individual species must lie elsewhere.

While it is not possible to say that the preponderance of certain combinations of α, β and γ is not due to natural selection, there is at present no evidence in favour of this hypothesis and the exploration of other possible explanations of the situation in *Elaphoglossum* is fully justified.

The significance of the association between certain grades of the characters taken two at a time.

(i) *Theoretical considerations.* If, after evolution has been occurring for some time in a genus, there remain several species close to the ancestral species in a group of characters, then, if the characters are graded, the primitive grades will necessarily be found more frequently in these species than in others. The primitive grades will not, therefore, be distributed evenly throughout the genus, but they will tend to be associated in the primitive species. Similarly, in the species farthest removed from the ancestral ones the primitive grades will be rare and will occur less frequently than would be expected were the distribution even. The most recent grades, on the other hand, will tend to be represented more frequently than would be expected in these species.

If, now, it be found in a genus, the phylogeny of which is not known, that there is a clear tendency for the grade of a character, for the primitiveness of which there is independent evidence, to be associated with certain definite grades of other characters, it follows, if these characters are independent and

if the association possesses no selective advantage, that the group of species containing this association is closest to the primitive state in respect of these characters. In these conditions, therefore, the grades of the characters associated with the one grade which is known to be primitive will be themselves primitive. In other words, a combination containing a primitive grade which occurs in a genus more frequently than would be expected can be taken, in these circumstances, to consist entirely of primitive grades.

This is a development and an adaptation to the particular problem presented by *Elaphoglossum* of the general method introduced by Frost (1930*a*, 1930*b*, 1931) and Kribs (1934–5), and used in a limited form by Sporne (1948) in an attempt to assess the primitiveness of certain characters in Angiosperms. The just criticisms made by Stebbins (1951)[1] of Sporne's hypothesis do not apply to the present treatment, since the possibility of natural selection accounting for the morphological picture in *Elaphoglossum* has been considered and regarded as unlikely.

(ii) *The association of* α_1 *with* β_3. In both American samples there is a clear association between α_1 and β_3. As will be seen from Table V (in Part I of this paper), the tendency for α_1 and β_3 to be associated is present also in the species of the Old World. Evidence has already been presented for the antiquity of α_1. It follows that β_3 is the primitive grade of β. The simplest hypothesis is that β_1 is consequently the most recent and β_2 intermediate. Further evidence on this point will be presented later.

(iii) *The association of* β_3 *with* γ_2. The combination $\beta_3\gamma_2$ occurs much more frequently than expected in both samples. It follows from the preceding that γ_2 is the most primitive grade of γ. The simplest hypothesis is that the phylogenetic series in γ dichotomizes, γ_1 being derived from γ_2 by simplification and γ_3 and γ_4 by elaboration, γ_4 being the most recent grade. This is also discussed further below.

(iv) *The association of* α_1 *with* γ_2. Although, as stated earlier, the combinations of α_1 and γ_2 are not such as to indicate dependence conclusively, the frequency of $\alpha_1\,\gamma_2$ in both samples exceeds the expected value. An association between these grades follows inevitably from the two preceding associations.

(v) *The status of* γ_1 *and* γ_3. If both γ_1 and γ_3 are one grade removed from γ in the same temporal sequence, as is proposed above, they can be regarded as belonging to one phylogenetic grade. They will also tend to have the same distributions with respect to α and β; this expectation is clearly fulfilled (Fig. 9) and attention has been drawn to it in earlier paragraphs.

The combinations of the characters taken three at a time. Working from the hypotheses, for which evidence has been presented, that the phylogenetic series in the characters α, β and γ are

$$\alpha_1 \rightarrow \alpha_2 \rightarrow \alpha_3 \rightarrow \alpha_4$$

$$\beta_3 \rightarrow \beta_2 \rightarrow \beta_1$$

$$\text{and } \gamma_2 \begin{cases} \nearrow \gamma_3 \rightarrow \gamma_4 \\ \searrow \gamma_1 \end{cases}$$

[1] Reference should also be made to a spirited reply by Sporne (1954).

it is possible to study the combinations of the phylogenetic grades three at a time. The frequencies of α_3 and α_4 are summed as before because of the smallness of their numbers, and the frequencies of γ_1 and γ_3 because these are now regarded as belonging to one phylogenetic grade. The frequencies with which the different combinations occur can again be compared with those expected, assuming the characters to be independent. The value of χ^2 for the Ecuado-

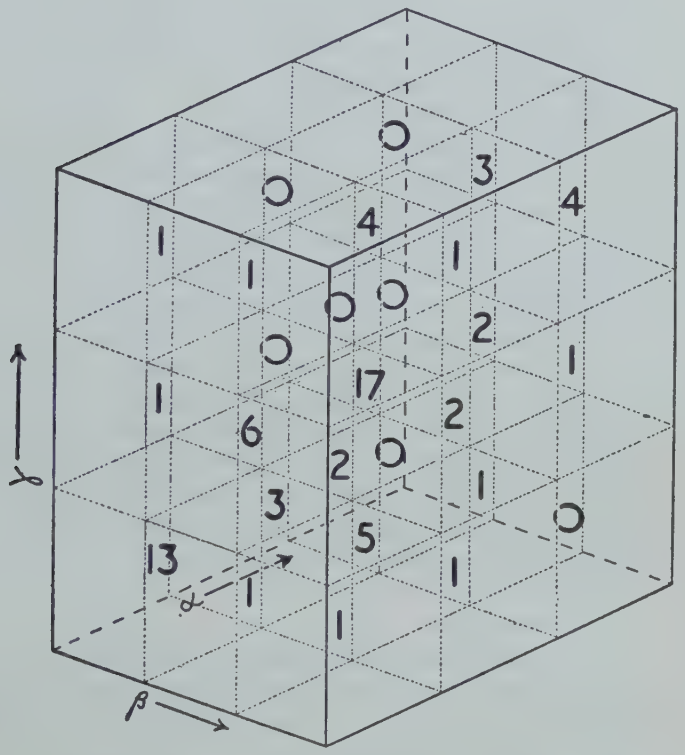

FIG. 10. The combinations of α, β, and γ considered simultaneously (combined American data). The frequencies are plotted according to the phylogenetic series in these characters, so that the cell at the origin has the composition $\alpha_1\beta_3\gamma_2$. For further explanation see text.

rean sample is 39·10. There are 8 degrees of freedom and the probability of obtaining such a value of χ^2 were the characters independent is less than 0·01. The deviation from the expected values is clearly significant.

It has already been seen that the Jamaican and Ecuadorean samples show similar tendencies. The homogeneity of the data is further shown by the three-dimensional analysis of the combined American data (Fig. 10). (*E. apodum* and *E. tectum*, which occur in both samples, have been counted only once.) The value of χ^2 is considerably greater than that given by the Ecuadorean sample alone and the probability of α, β and γ being distributed independently is so small that it can be neglected. ($\chi^2 = 87{\cdot}31$, $P < 0{\cdot}001$.)

The partitioning of χ^2. The greatest contribution (42·76) to the value of χ^2 is given by the cell $\alpha_1\beta_3\gamma_2$, revealing a strong association between these grades. This supports the argument that they are all primitive grades.

It will also be observed that the combinations $\alpha_1\beta_1\gamma_4$, $\alpha_{3+4}\beta_3\gamma_4$, and $\alpha_{3+4}\beta_1\gamma_2$ have not been recorded at all. These are the combinations in which one primitive grade occurs with what are believed to be the most recent grades of the other two characters. The fact that these combinations have not appeared supports the hypothesis of the recentness of α_4, β_1 and γ_4 since, owing to the rarity of primitive grades in recent species, the greater the number of recent grades taken together the smaller will be the chance of finding a primitive grade in association with them.

It follows that the combination $\alpha_4\beta_1\gamma_4$ is composed entirely of recent grades and, in respect of these characters, the group of species showing it is the most recent in the genus. This is further supported by the fact that this combination also occurs more frequently than expected and gives the second greatest contribution (25·62) to χ^2. It has already been argued that the most recent grades will not be evenly distributed, but will tend to be associated in recent species. That such an association of α_4, β_1 and γ_4 does in fact occur is then a further indication of their recentness. It is significant that the species showing the combination $\alpha_4\beta_1\gamma_4$, the only species in which α_4 occurs, so far as can be discovered, have such a restricted geographical distribution.

The combinations $\alpha_2\beta_2\gamma_{1+3}$ also occur more frequently than expected, but their contribution (7·95) to χ^2 is much less than that of $\alpha_1\beta_3\gamma_2$ and $\alpha_{3+4}\beta_1\gamma_4$. It is doubtful whether much significance can be attached to their over-representation, since the middle grade of each series contains a greater anatomical range than either of the extremes, a range which it is impossible to subdivide and grade with any precision.

In conclusion, it can be stated that the statistical analysis of the occurrence of α, β and γ, taken together with the differences in the geographical distributions of the grades of α, points to the primitiveness of α_1, β_3 and γ_2 and to the recentness of α_4, β_1 and γ_4. The argument presented in support of this view is consistent and it has not been necessary to make any assumptions other than those set forth at the beginning in the theoretical considerations of the problem. It will now be shown that this knowledge of certain evolutionary progressions in *Elaphoglossum* permits the evaluation of the phylogenetic status of other morphological features and is in accord with what is so far known of the geographical distribution of the genus.

The Phylogenetic Significance of other Morphological Features

The freely branching forms. The species with the freely branching rhizomes have already been listed (Part I, p. 176). In the Old World this condition appears to occur only in *E. bolanicum* Ros., confined to New Guinea, and possibly in *E. angulatum* (Bl.) Moore, more generally distributed in SE. Asia. It is, therefore, a character more frequent in the New World than in the Old,

but probably not proportionately so. Five of the ten freely branching species examined possess the primitive combination $\alpha_1\beta_3\gamma_2$. Of the rest, *E. nematorhizon* and *E. procurrens*, each with α and β in the primitive condition, show other than the primitive grade of γ. *Bell 665* shows α and γ in the primitive condition combined with the most recent grade of β. Both *Bell 97* and *Bell 100* show the primitive grade of α combined with more recent grades of β and γ.

The statistical test applied to the combined American data shows no significant association between the branching condition and the primitive combination of α, β and γ, nor, on the other hand, is there any significant association between the unbranched condition and this combination. The freely branching forms appear, therefore, to have arisen at various times in the evolution of the genus.

All the freely branching species examined have possessed α_1 steles, but this may be due to an obligate developmental relationship between the two-ranked condition, the length of the internodes and the capacity of the lateral buds to develop. This has already been discussed from a morphological point of view (Part I, p. 179) and these relationships can now be given a phylogenetic interpretation. This is considered further below.

Hydathodes and other features. A list of the species in which the lateral veins of the lamina end in hydathodes has also been given earlier (Part I, p. 192). Reference to Table V shows that there is a conspicuous predominance among these species of the more recent grades of α, β and γ. There is no obligate morphogenetic connexion between hydathodes and the more recent grades of these characters, nor any evidence of selective advantage bestowed by their presence. Hence the development of hydathodes can be regarded as a recent feature in the genus.

Similar reasoning leads to the view that the long narrow scales found on the rhizomes of some species (Part I, p. 192), the tendency for the stoma to be enclosed by an epidermal cell (Part I, p. 198) and the failure of the central metaxylem tracheids to be lignified in the emerging root trace (Part I, p. 198) are also features which have arisen in the more recent evolution of the genus.

Since γ_3 scales have now been shown to be more recent than γ_2, and since γ_2 scales have never been found occurring in dense mats, it is all the more probable that leaves with a dense investment of γ_3 scales have been derived from those with more scattered scales. The changes of stomatal index in relation to scaliness discussed earlier (Part I, p. 194) may therefore have a phylogenetic significance.

The Nature of the Phylogenetic Trends in *Elaphoglossum*

The evolutionary changes at the apex of the rhizome. The conclusion to be drawn from the phylogenetic series which has been demonstrated in the stele of *Elaphoglossum* and the associated changes in the development of the internodes and the lateral buds seems inescapably to be that the lateral buds in this genus represent a potential branching system which has been inherited

from the ancestors of the genus. In the evolution of the stele from its initial two-ranked condition there appear to have been two changes which have taken place at the apex; firstly, an increase in the rate of initiation of leaf primordia relative to extension growth, with the result that dorsiventrality has eventually been eliminated and the apex has become negatively geotropic, the internodes have become very much shortened, and the development of the buds has been almost totally suppressed; secondly and less commonly, a diminution in the rate of initiation of leaf primordia relative to extension growth, giving rise to the few species in which the branching system is given its full expression.

This is contrary to the views of Wardlaw (1952), who maintains that the position and development of buds in ferns is independent of the hereditary constitution of the plant. The lateral buds investigated in *Elaphoglossum*, however, which have directed towards them a well-developed vascular strand from the stele (Bell, 1951), whether they be dormant or not, are clearly of a different nature from the superficial buds lacking a vascular connexion which Wardlaw has investigated in *Matteuccia* and elsewhere. It is interesting to note that Street (1954) has been able to control the extent of branching in roots cultured *in vitro* and the whole morphological pattern developed by them by varying the amount of anti-auxins in the medium. Similar experiments have not yet been performed on shoots, but it may be that the pattern developed by the rhizome of *Elaphoglossum* depends upon the effective balance of auxin in it, this balance being constant for the mature form of each species, but differing between species.

The genetic control of stelar form has still to be investigated, since it has not yet been possible to attempt the hybridization of appropriate species of *Elaphoglossum* and study the structure of hybrids of the first and subsequent generations. The series of stelar forms may be due either to co-ordinated changes in a number of genetic factors or to the existence of a series of allelomorphic genes acting at one locus. A morphological series of leaves from entire to lobed is known to be determined by such a series of alleles in *Gossypium*. The developmental study of the leaves of a number of species of this genus (Stephens, 1944) reveals remarkable parallels to the situation in the stele of *Elaphoglossum*. The developmental system here, considered generically, can be seen to consist, as does that of the leaves of *Gossypium*, of a common track divided into three phases, these being in *Elaphoglossum*: phase A, the two-ranked condition: phase B, states intermediate between the two-ranked and the radially symmetrical condition: phase C, the radially symmetrical condition. Species showing α_1 steles have remained in phase A, while those showing α_2 and α_3 steles have reached phase B, although, as pointed out earlier (Part I, p. 178), these species initially spend a varying amount of time in phase A. Species with α_4 steles have attained phase C, but in their ontogeny phases A and B are missing, although the whole series is shown almost complete in *Bell 765*. In *Gossypium*, Stephens found that the succession of leaves in species in which the mature form was of the final phase also showed very little of the preceding phases.

These similarities may be merely superficial, but they are sufficiently striking to suggest that the stelar series in *Elaphoglossum* may also be due to a series of allelomorphic genes. This locus would then be responsible for controlling, possibly by influencing the balance of antagonistic hormones, the relative rates of leaf initiation and extension growth at the apex of the rhizome. A series of mutations at this locus would account for the morphological series of forms, but there would be no question of an orthogenetic trend. The telescoping of the early phases in the development of the most recent form of stele may be due to mutations at other loci controlling the time of inception of the phases of the developmental track at the apex.

The evolutionary changes in the petiole and the scales of the frond. These, too, may be due to changes in several genes or in one. No phasic development has been observed in these characters in the ontogeny of the plant. The elucidation of their genetic control, like that of the stele, awaits the accumulation of evidence from experimental breeding.

The Relation Between the Geographical Distribution of *Elaphoglossum* and its Phylogeny

Although the distribution of the different morphological forms of *Elaphoglossum* is still imperfectly known, sufficient has been discovered in this investigation to reveal marked differences in the *Elaphoglossa* of areas of different geological age.

The primitiveness of the Malayan species. Of the six species known from the Malayan peninsula, five show the primitive combination $\alpha_1\beta_3\gamma_2$. In SE. Asia generally the primitive combination is predominant. It is significant that two families of ferns, the Matoniaceae and the Dipteridaceae, both well represented and widely distributed in Mesozoic times, are today confined to this region. Other ancient ferns, such as the Marratiales, the Schizaeaceae and the Gleicheniaceae, are also frequent. According to Arldt (1919), the continental area of SE. Asia has presented a continuous land surface since before the end of the Mesozoic period. Since there is no evidence of major volcanic activity or climatic change in the region during Tertiary or recent times, the possibility arises that the mountains have known a continuous forest cover for the greater part of the time during which *Elaphoglossum* as a genus has been in existence. In such a closed environment, presenting few new surfaces for colonization, evolution may be expected to be slow. The primitiveness of the *Elaphoglossa* of the Malayan peninsula is therefore in accord with the geological and climatic history of the region.

The origin of the pan-tropical distribution. Although Polunin (1951) has shown that the spores of the Pteridophyta can reach the upper air currents, it is not known whether the pan-tropical distribution of certain genera can be accounted for in this way. If the existence of former land bridges is to be invoked, the mountainous isthmian links for which Willis (1932) has advanced detailed geological and climatological arguments might well account for the

occurrence of *Elaphoglossum* throughout the Tropics. It is generally held that these bridges were submerged during the Cretaceous (Caster, 1952).

If the spread of *Elaphoglossum* has taken place along such mountain bridges, then the surviving ancient mountains of the Tropics, which were formerly linked by them, might, if there is no evidence of their having been disturbed by climatic change or volcanic activity, still be carrying, for the same reason as the Malayan peninsula, a relatively primitive *Elaphoglossum* flora. The data to test this hypothesis are, unfortunately, very few, but some which support it are available from South America. The Highlands of Eastern Brazil and the Guiana Highlands of Venezuela, North Brazil, and Guiana are believed to be the remains of an ancient mountainous system (Weeks, 1947). The Sierra de la Macarena of eastern Colombia is also believed to have formed part of this system (Schultes, 1954), and the flora of these mountains, although they have hitherto been little explored, already shows significant affinities with that of the Guiana Highlands and the mountains of Eastern Brazil (Alston, 1952). The conditions existing in the forests of the upper part of the Sierra de la Macarena are not dissimilar to those in comparable parts of the adjacent Andes, yet only three species of *Elaphoglossum* were collected by Philipson, Doncaster, and Idrobo (Alston, 1952) and no more were found by Bell (1953). These three species, *E. aphlebium*, *E. eximium*, and *E. scalpellum*, each show β and γ in the primitive condition. The first and the third also show α in the primitive condition. *E. eximium* reaches the α_2 grade. This suggests that in these mountains, too, *Elaphoglossum* may have evolved only slowly and that the environment may be one of long standing. It is significant that the neighbouring and much more recent Andes have such a diversity of species, the greater proportion of which are advanced in respect of α, β and γ.

Volcanic activity as a stimulus to evolution. Volcanic activity, as well as the mountain building of the Tertiary period, has provided an abundance of new habitats in the Tropics. Such activity may have contributed to the acceleration of evolution in *Elaphoglossum* in the Andes, since much of the mountain forest is on the slopes of volcanoes. It is noteworthy, too, that the African *E. clarenceanum* (Fernando Po and San Tomé), *E. kuhnii* (Cameroons), and *E. phanerophlebium* (Madagascar), all of which are outstanding Old World species in being advanced in respect of α, β and γ, occur in areas that have been subject to volcanic activity in late Tertiary or recent times.

Isolation as a stimulus to evolution. According to Schuchert (1935), the whole of Jamaica was probably submerged in the Oligocene. Recolonization of the island took place during the Miocene, during part of which period there was a land bridge connecting the island with Honduras. Whether the invading *Elaphoglossa* came by long distance air dispersal or by short stages across the land bridge, they would be representative of a flora which was no longer primitive, since the new land surfaces which had begun to arise and which eventually formed the Andes and the mountains of Central America (Weeks, 1947) would have encouraged the evolutionary advance of the genus. The subsequent total or partial isolation of the *Elaphoglossum* flora of the

island following the faulting of the Pliocene and the subsidence of the land bridge might have caused, as is known to occur in small populations, the appearance of new forms more rapidly than amongst the flora of the mainland. This may account for the much higher proportion of species with the advanced combination $\alpha_4\beta_1\gamma_4$ occurring in Jamaica than in Ecuador. The detailed study of other island floras is necessary to substantiate this point.

Summary and Conclusions

1. Morphological series are demonstrated in the forms of the stele, the structures at the base of the petiole and the scales of the lamina of the frond in *Elaphoglossum*. Each series is graded and each character is given a symbol to facilitate reference, the symbols being α, denoting the stele, β, the petiole, and γ, the scales of the frond. The material upon which these results are based has been drawn from several localities in the Tropics of the Old and New Worlds.

2. The stomatal frequencies of the American species have been estimated and it appears that a very dense scaliness of the lower surface of the frond is accompanied by a high stomatal frequency. No close relationship between scaliness and habitat was observed in the field. A very careful comparison of two pairs of species, the members of each pair growing together in the same habitat, leads to the view that the association of high stomatal frequencies with dense scaliness may result from nutritional factors affecting the developing epidermis.

3. The genetical significance of the morphological series is discussed and the conditions in which the genus has probably evolved are reviewed. It is concluded that there is no reason why the initial states of the stele, petiole, and scales should not have persisted in the genus. The morphological series are therefore phylogenetic series, but the phylogenetic order, which is not necessarily the same as the morphological, has to be decided in the light of other evidence.

4. Eighty-seven species of *Elaphoglossum* have been analysed according to the grades of the characters α, β and γ displayed in their mature forms. The representation of the different combinations of α, β and γ has been determined in the American collections. It is found that certain combinations of the grades occur much more frequently than would be expected were the characters independent. These grades are said to be associated.

5. Three possible explanations of the associations of certain grades are considered, namely: (i) that the characters are developmentally or genetically connected so that there are obligate relations between them; (ii) that the combinations occurring more frequently than expected possess some selective advantage which enhances their representation; and (iii) that the grades of the more frequent combinations are either primitive or recent.

From this consideration it is concluded: (i) that there is no evidence that the characters are developmentally or genetically related; (ii) that there is no evidence that the more frequent combinations possess any selective advantage;

and (iii) that, provided that the characters are not genetically or ontogenetically linked and provided no combinations possess a selective advantage, there are theoretical grounds for expecting the primitive and the recent grades of each character to be associated.

6. Independent evidence is presented for the antiquity of α_1. Because of their association with α_1 it is argued that β_3 and γ_2 are also primitive. The morphological series in α, β and γ are therefore rearranged according to the probable phylogenetic sequence in these characters. The variation in the three characters is now considered simultaneously, the frequencies being plotted in the order of these phylogenetic sequences. It is found that the distribution of the grades is clearly not even. The combination of the primitive grades occurs very much more frequently than expected, as also does that of the grades believed to be the most recent. This agrees with the distribution predicted on theoretical grounds and supports the validity of the phylogenetic sequences. The homogeneity of the Ecuadorean and Jamaican data is shown by the fact that when they are summed the statistical test is more conclusive than when the Ecuadorean data are considered alone.

7. The phylogenetic status of certain other features is considered.

8. The nature of the evolutionary changes in *Elaphoglossum* is discussed. It is shown that there are marked parallels between the phylogenetic series in the stele and the sequence of leaves in species of *Gossypium*, where leaf shape is known to be governed by a series of allelomorphic genes. It is suggested that a series of allelomorphs may determine the mature form of the stele in *Elaphoglossum*.

9. It is shown that certain mountainous regions of the Tropics which have probably borne a cover of forest since Mesozoic times possess a primitive *Elaphoglossum* flora. Other areas known to have been recently created or disturbed possess floras containing a high proportion of recent species.

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Studies in the Physiology of Parasitism

XXI. The Production and Properties of Pectic Enzymes secreted by *Fusarium moniliforme* Sheldon

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ABSTRACT

Fusarium moniliforme secreted macerating enzymes in liquid media only when these contained certain natural extracts, pectic substances, or galacturonic acid. Apple extract was unsuitable for enzyme secretion and also inhibited enzyme secretion in synthetic media otherwise suitable.

Protopectinase activity of solutions was highest in the pH range 8·0–9·0, was rapidly lost at temperatures above 50–60° C., and was reduced by concentrations of phosphate higher than 0·02 M. The enzyme was partially purified by precipitation in 60 per cent. acetone at pH 6·0.

Protopectinase solutions also contained an enzyme which reduced the viscosity of solutions of various pectic substances. The properties of this enzyme were, in general, similar to those of protopectinase.

When activity of enzyme solutions was measured by the liberation of reducing groups, pectate solutions were more rapidly degraded than were solutions of a high methoxyl pectin, particularly in the early stages of the reaction. Paper chromatography of the products formed showed that pectate and pectin were degraded in different ways.

Although the pathogen readily secreted protopectinase in potato extract, potato tubers were not readily parasitized. In contrast, *Fusarium avenaceum* which readily attacked tubers, secreted little protopectinase in potato extract.

I. INTRODUCTION

THE fungus *Fusarium moniliforme* Sheldon is an economically important parasite of a considerable number of tropical and sub-tropical plants. It is perhaps best known as a pathogen of various grasses and, in particular, of sugar cane in which it causes the disease known as top-rot; it has also been reported on such miscellaneous hosts as potato tubers, apple and tomato fruit, and the cotton plant. Because of the general nature of its parasitism and the ease with which it is cultured, this fungus was considered a promising subject for physiological studies and for the study of pectic enzymes since it typically causes a soft rot of infected plant tissues.

The work described in this paper is concerned chiefly with an analysis of the nutritional factors affecting the production of macerating enzymes, that is, of enzymes which produce a typical soft rot of susceptible plant tissues, and with the properties of these and certain other pectic enzymes. The general background of the work has already been summarized in a recent paper of this series (Wood, 1955), which also outlined the nomenclature of the pectic substances and enzymes to be used in this paper.

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II. Experimental Materials and Methods

The culture of *F. moniliforme* which was used was obtained from the Indian Agricultural Research Institute and had been isolated originally from sugarcane. Stock cultures were maintained on potato-extract agar on which good growth and sporulation was obtained; they were transferred to fresh media at monthly intervals and, as far as could be seen, remained constant in behaviour and cultural characteristics. It may be noted in passing that cultures kept at 4 or −16° C. for 18 months remained viable.

The citrus pectin, pectic acid, and sodium polypectate (sodium ammonium pectate) used in the course of the work was supplied by the California Fruit Growers Exchange, Ontario, California, and was washed before use in acidified ethanol (5 vol. concentrated HCl, 35 vol. water and 60 vol. ethanol), in 60 per cent. (v/v) ethanol until free of chloride and finally in 90 per cent. ethanol. It was then dried under vacuum and stored at 4° C. in airtight containers.

Liquid cultures were grown in 8 oz. medicine bottles containing 20 ml. of medium. When synthetic media were used, phosphate was sterilized separately from the other constituents. Media were sterilized by autoclaving for 15 minutes at 15 lb. pressure and inoculated with 1 ml. of a dense spore suspension. Cell-free filtrates were prepared from cultures, normally after 4–5 days' growth at 25° C., by passing through muslin and centrifuging at 10,000 r.p.m. for 10 minutes; they were stored at −16° C. until required.

The dry weight of mycelium from cultures was obtained by collecting the washed mycelium on a weighed filter paper and drying at 70° C. for 24 hours. The figures given are the means of three determinations.

Macerating activity was estimated by placing three discs (8 mm. diam., 0·5 mm. thick) of fully turgid potato tuber tissue in the test solution and determining the times (Reaction Times = R.T.) at which coherence was lost when the disc was subjected to slight tension by pulling from opposite sides. Macerating tests were carried out at known temperatures and pH values. The pH values of the solutions were generally adjusted with 0·1 N NaOH or 0·1 N HCl and maintained at the required value with veronal buffer (Michaelis, 1930) unless otherwise stated. In each test 0·5 ml. of the buffer was added to 2·5 ml. of the enzyme solution.

The activity of the enzyme, which reduced the viscosity of solutions of pectic substances, was measured from periodic viscosity determinations made with an Ostwald viscometer at 25° C. Details of the substrate and the methods used for assaying activity will be given later.

III. Effect of Nutrient Conditions on Secretion of Protopectinase

(*a*) *Natural media.* The fungus was grown on the following natural media for 5 days at 25° C.: tomato, potato, turnip, and apple extracts; 200 g. of these tissues were boiled separately, passed through muslin and made up to 1 litre.

A tomato pulp made by diluting a commercial preparation ten or twenty times was also used.

Cell-free filtrates of cultures were adjusted to and maintained at pH 8·0, the optimal value for enzyme activity (see later), and the macerating activity determined. The results are given in Table I.

All media except apple extract were suitable for enzyme production and became alkaline during growth of the fungus. Although apple extract supported a reasonable growth, the culture filtrate had little if any protopectinase activity. A feature of the results of this and of many similar experiments was the absence of a clear relation between growth and enzyme secretion.

TABLE I

Secretion of Protopectinase on Natural Media

Medium	pH		R.T. (Min.)	Dry wt. mycelium (mg.)
	Initial	Final		
Tomato extract	5·1	8·7	40–45	212
Potato ,,	7·1	8·6	80–90	165
Turnip ,,	6·4	8·2	130–50	138
Apple ,,	3·8	6·2	> 1440	100
Tomato pulp 5%	4·8	8·6	110–20	118
,, ,, 10%	4·6	8·8	40–50	210

In further experiments with tomato pulp which seemed particularly suitable for enzyme production, the activity of filtrates increased with the concentration of the pulp in the medium; after growth in undiluted pulp, the R.T. of filtrates was as low as 25–30 minutes. When tomato-pulp preparations were clarified by centrifuging at 10,000 r.p.m. for 10 minutes and the supernatant solutions used as culture media, growth and pH changes were similar to those in untreated preparations but the enzyme activity was negligible. The possible significance of this result will be referred to later.

Active enzyme preparations were also obtained when the juice pressed from tubers which had been frozen was used in various dilutions. Much less active solutions were obtained from cultures on juice from frozen turnips; apple juice obtained in the same way supported good growth but filtrates were inactive.

The addition of 0·5 per cent. (w/v) glucose or asparagine in the absence or presence of 1 per cent. $CaCO_3$ to various dilutions of apple juice had no effect on enzyme secretion. Similar results were obtained when dialysed juice was used. These data suggest that apple juice contains one or more substances which prevent secretion of the enzyme or reduce its activity after secretion; evidence for this is given later.

(*b*) *Synthetic media.* The following six media were used:

(i) Glucose 1·0%, asparagine 0·5%, KH_2PO_4 0·1%, $MgSO_4.7H_2O$ 0·05%.

(ii) Glucose 0·5%, starch 2·5%, asparagine 0·5%, K_3PO_4 0·03%, $MgSO_4.7H_2O$ 0·02%. (Ashour (1949) for production of protopectinase by *Pythium debaryanum*.)

(iii) Sucrose 3·0%, $NaNO_3$ 0·2%, K_2HPO_4 0·1%, $MgSO_4.7H_2O$ 0·05%, KCl 0·05%, $FeSO_4.7H_2O$ 0·001%.

(iv) Glucose 1·0%, peptone 0·5%, KH_2PO_4 0·1%, $MgSO_4.7H_2O$ 0·05%.

(v) Glucose 0·2%, starch 4·0%, asparagine 0·18%, peptone 0·18%, K_3PO_4 0·14%, $MgSO_4.7H_2O$ 0·08%, KCl 0·015%, $FeCl_3.6H_2O$ trace. (Synthetic potato medium after Brown, 1925.)

(vi) Glucose 0·2%, asparagine 0·2%, K_3PO_4 0·13%, $MgSO_4.7H_2O$ 0·08%.

Growth was good in each with the final pH values falling in the range 7·8–9·2; enzyme secretion was, however, poor in all. The effect of adding a natural extract to one of these media was therefore studied. Tomato pulp was added in various concentrations to the following medium: glucose 1·0%, asparagine 0·5%, KH_2PO_4 0·1%, $MgSO_4.7H_2O$ 0·05%. The enzyme

TABLE II

Effect of Citrus Pectin on Protopectinase Secretion

Medium	pH		R.T. (Min.)	Dry wt. of mycelium (mg.)
	Initial	Final		
(i) Glucose-asparagine	5·4	8·2	1,200	153
" " +1% pectin	3·5	8·6	20–25	196
(iii) Sucrose-nitrate	7·2	7·8	1,200	159
" " +1% pectin	3·8	5·3	240	181
(iv) Glucose-peptone	5·8	7·8	1,200	208
" " +1% pectin	3·6	5·2	300–60	209
(v) Glucose-starch-asparagine	7·4	7·8	270–300	272
" " " +1% pectin	6·2	5·4	100–10	292

activity of the filtrates from the different media was clearly related to the amount of tomato pulp which had been added and was negligible in its absence.

Some micro-organisms are known to secrete pectic enzymes adaptively and as tomato pulp might be expected to contain relatively large quantities of pectic materials the effect of adding a citrus pectin to synthetic media (i), (iii), (iv), and (v) above was investigated. The results are given in Table II.

The addition of pectin greatly increased enzyme secretion in each case; in three media, this was associated with some although not a large increase in growth. The most active preparation was obtained with the glucose-asparagine medium to which pectin had been added; this was also the only pectin containing medium in which an alkaline reaction developed.

Similar experiments were carried out with potato, turnip, and apple extracts. The addition of pectin slightly increased growth but had little effect on enzyme secretion; quite active preparations were again obtained with potato extract, turnip extract filtrates had only low activity while those based on apple extract were inactive. Apple extract was also found to inhibit enzyme secretion when added to synthetic media highly suitable for enzyme production. The production of protopectinase would therefore seem to depend on the presence of pectic substances in the culture media. This probably explains the suitability

of tomato pulp for enzyme production and why clarification of tomato pulp made it unsuitable, as much if not all the pectic material in the original pulp would have been removed by centrifuging.

In view of the suitability of the glucose-asparagine medium, a detailed analysis was made of the effect of the different constituents on enzyme secretion: the most active preparations were obtained from cultures on the following: glucose 0·5%, pectin 1·0%, asparagine 0·4%, KH_2PO_4 0·1%; $MgSO_4.7H_2O$ 0·05%; excess $CaCO_3$ (1%) was also added to maintain a suitable pH for growth from the beginning. This medium was used extensively for the preparation of enzyme solutions used in work described later. Cell-free filtrates of cultures grown on it for 5 days at 25° C. will be referred to as standard enzyme solutions.

A number of other carbon sources were tested instead of glucose in the above medium; fructose, sucrose, and maltose gave preparations of activity similar to that obtained with glucose, while lactose, starch, and cellulose gave preparations of somewhat lower activity. Mycelium production was of the same order in each case.

Replacement of asparagine with peptone, sodium, or potassium nitrate, ammonium chloride or ammonium nitrate (as equivalent N) in unbuffered media resulted in poor growth and enzyme secretion. This was probably caused by the inability of the fungus to modify the pH of the media, which was initially about 3·0, to values favourable for growth, as *F. moniliforme* grows poorly at low pH values. In the presence of 1 per cent. $CaCO_3$, active preparations and good growth was obtained with each nitrogen source tested; filtrates from asparagine and peptone media had R.T. values of 15–20 minutes; filtrates on the other media were less active with R.T. values in the range 30–70 minutes.

Pectin was also replaced by a variety of pectic or allied substances or by galacturonic acid, each at a concentration of 1 per cent. Active preparations were obtained with a protopectin prepared from turnip roots, sodium pectate, sodium polypectate, and with galacturonic acid. Filtrates had little or no activity when pectin was replaced by galactose, gum arabic, gum tragacanth, alginic acid, or mucic acid. It was apparent that only pectic materials or one of their breakdown products, galacturonic acid, were suitable for enzyme secretion; it may be added here that the fungus did not grow well in unbuffered media containing galacturonic acid. This was probably caused by the low initial pH (3·1) of these media; enzyme secretion was also poor in these conditions. In further studies on the effects of different proportions of glucose and galacturonic acid it was found that maximum activity was obtained with 0·5 per cent. glucose and 1 per cent. galacturonic acid and that there was no enzyme secretion in the absence of the acid. Once again, there was little relation between enzyme secretion and growth of mycelium.

In the above experiments, only the extra-cellular enzyme was considered. Protopectinase could also be prepared from the mycelium, as was shown by the following. Mycelia were collected from twelve cultures, that is from

approximately 240 ml. of the standard medium described earlier. They were washed thoroughly with water and pressed between absorbent paper to remove surplus liquid. The mycelia were placed in acetone at −18° C. for 30 minutes, the acetone was then removed and the mycelial mat dried under vacuum; the dried mycelia, weighing 1·5 g., were ground finely with sand and suspended in 50 ml. of water and kept overnight at 4° C. The suspension was then cleared by centrifuging and the supernatant liquid removed. The pH of the final solution was 6·8; it was adjusted to pH 8·0 before testing for activity with potato tuber discs. The reaction time was 15–20 minutes, that is, the activity was of the same order as that of the extra-cellular enzyme in the culture filtrates.

IV. Properties of Protopectinase

(*a*) *Dilution.* A standard enzyme solution was diluted with different amounts of an autoclaved solution and tested for enzyme activity in the usual way. Activity, expressed at 1000/R.T.(min.), was plotted against enzyme concentration to give a dilution curve which was sometimes used when it was required to estimate the effect of certain treatments on the activity of the standard enzyme solution. It was assumed that the latter had an activity of 100 arbitrary units which corresponded to an R.T. of 15 minutes so that, for example, a sample of the standard enzyme solution which after a certain treatment gave an R.T. value of 20 minutes would be assumed to have an activity of 60 arbitrary units.

(*b*) *Thermal inactivation.* Five ml. samples of the standard enzyme solution were transferred to thin glass vials in water-baths at different temperatures. After 2 or 5 minutes at a particular temperature, they were placed in ice-water. When all samples were ready, the activity of each was tested using slices taken from one tuber; the results showed that with 5 minutes' exposure, inactivation was rapid over the range 40–60° C., then more gradual until at 90° C. only some 5 per cent. of the original activity remained. With 2 minutes' exposure, there was considerably less inactivation up to 70° C.; above this temperature the curves for 2 and 5 minutes' exposures came together. Solutions were completely de-activated after 20 minutes at 100° C. or by autoclaving at 120° C. for 15 minutes.

(*c*) *Effect of* pH. Samples of the standard enzyme solution were brought to a series of pH values in the range 2·0–10·0; veronal buffer was added and the activity of the solution estimated from measurements of reaction times. The pH values of the solutions were taken at intervals during maceration and also when maceration was complete. Only in a few samples did the pH value remain constant, in all others changes of pH occurred during the tests; these were slight except at the ends of the range where they were as much as 0·5 units. The optimum pH was in the range 8·0–9·0; activity fell rapidly on either side of this range. There was little or no activity at pH 5·0 and no activity was detected at pH 2·0 after 24 hours. All discs lost their turgidity

below pH 5·0, but there was no maceration in the controls even at the lowest values.

(*d*) *Effect of dialysis.* Samples of a standard enzyme solution were dialysed, first in running tap water for 6 hours and then in distilled water at 4° C. for 18 hours. After correcting for volume changes during dialysis, the macerating activity of these samples was compared with samples which had been kept at 4° C; the samples had very similar activities when tested at different dilutions.

(*e*) *Effect of phosphate.* Concentrations of phosphate up to 0·02 M at pH 8·0 had little effect on activity. Above this concentration, however, activity was reduced rapidly and at a concentration of 0·1 M there was little or no activity.

(*f*) *Effect of storage.* Samples were kept under toluene at various temperatures for 1, 5, and 10 days. There was a fairly rapid loss of activity at 20 and 25° C., e.g. a 50 per cent. loss in 3 days at 25° C. At 4° C. there was little loss after 6 days but a 40 per cent. loss after a further 4 days, while at −16° C. no loss was detectable after 10 days. Further tests at −16° C. showed that solutions retained full activity for at least 3 months.

(*g*) *Precipitation.* Samples were precipitated from aqueous solution at known pH values with different concentrations of acetone or alcohol. The latter were cooled to −16° C. before adding slowly with constant stirring to the enzyme solution at 4° C. The pH of the solutions after adding the precipitant was adjusted to the original value where necessary. The precipitates which formed were collected after 2 hours' storage at 4° C. and then kept in a vacuum desiccator for 30 minutes to remove most of the acetone or alcohol. The precipitate was then dissolved in half the original volume of enzyme solution and the activity measured. The optimum pH for precipitation with acetone was 6·0; at this value some 70 per cent. of the original activity was retained. At pH 7·0, 50 per cent. recovery was obtained, while at other pH values recovery was much poorer. When ethanol was used, precipitation from solution at pH 8·0 gave best results. At the optimum pH values, maximum activity was obtained when the precipitants were used at a final concentration of 60 per cent. or above; precipitates obtained at lower concentrations had considerably less activity.

V. Properties of the Viscosity-reducing Enzyme

The pectolytic activity of preparations from cultures of *F. moniliforme* was also measured viscometrically. The details of the method used have been given in an earlier paper of this series (Wood, 1955).

Pectin is a very suitable material for viscometric assays, but the activity of the viscosity reducing enzymes from some organisms is influenced by the methoxyl content of the substrate (Jansen and MacDonnell, 1945). Before using pectin solutions, therefore, the pectinesterase activity of preparations from cultures of *F. moniliforme* were determined. Very little activity was

detected over a period of 2 hours at pH 8·0, which was near the optimum for the activity of the viscosity-reducing enzyme. As viscosity determinations normally lasted for only 25 minutes, there could have been little change in the methoxyl content of the substrate during this period even with pectin as a substrate. A pectin solution was therefore used in most of the work described below. The composition of the substrate used was as follows:

1·0 per cent. pectin solution 5·0 ml., 0·1 N NaOH 0·8 ml., veronal buffer at pH 8·0 2·0 ml., water 0·2 ml. To this mixture was added 2·0 ml. of an enzyme solution; a series of viscosity determinations was then made at intervals over a 25-minute period using an Ostwald viscometer at 25° C. Smooth curves were obtained when viscosity, represented by the time of flow of the test solution through the capillary of the viscometer, was plotted against time. The effect of diluting the standard enzyme solution was investigated by mixing appropriate volumes of untreated and autoclaved solutions and adding aliquots to the substrate given above. From the series of viscosity/time curves which were obtained, the time taken for a particular mixture to show a certain loss of viscosity could be determined. When preparations of *Bacterium aroideae* were used in the above manner (Wood, 1955), a linear relation was obtained when enzyme concentration was plotted against activity represented by the inverse of time taken by a mixture to show a given loss of viscosity; similarly Roboz, *et al.* (1952) have shown that a linear relation was obtained when the logarithm of enzyme concentration of *Neurospora crassa* preparations was plotted against activity represented by the percentage loss of viscosity of the mixture. These and similar methods did not give linear relations with the data obtained with *F. moniliforme* preparations. A calibration curve was therefore drawn which showed the relation between enzyme concentration and the percentage drop in viscosity 25 minutes after adding the enzyme. The percentage loss of viscosity was calculated as follows:

N_o = Viscosity at time o, N_w = Viscosity of water,
N_t = Viscosity at time t;

$$\% \text{ loss of viscosity at time } t = \frac{N_o - N_t}{N_o - N_w} \times 100.$$

It was found that the activity of enzyme solutions increased after dialysis; a calibration curve similar to the above was therefore obtained for measuring the activity of unknown dialysed preparations.

Some of the properties of the viscosity-reducing enzyme were as follows: (unless otherwise stated all tests used an 0·5% pectin solution at pH 8·0.)

(*a*) *Thermal inactivation.* Samples of undialysed and dialysed solutions were treated at various temperatures for 5 minutes and then held at 0° C. until tested. The activity of undialysed solutions was rapidly reduced by exposure in the range 40° C.–50° C. and almost completely lost after 5 minutes at 70° C. A slight recovery of activity was obtained after heating at 80° C., but activity, was further reduced at 90 and 100° C. The dialysed preparation was considerably

less affected by temperatures above 50° C., but otherwise behaved as did the undialysed solution. In contrast with the latter, some 20 per cent. of the original activity remained in the dialysed solution after 5 minutes at 100° C. Autoclaving at 120° C. for 15 minutes completely deactivated both preparations.

(*b*) *Effect of* pH. Pectin solutions were adjusted to different pH values with 0·1 N NaOH and veronal buffer before adding 1·0 ml. samples of dialysed or undialysed preparations. Both types of solutions behaved similarly with negligible activity at pH 3·0 and only a slight increase up to pH 7·0. Activity increased ten times between pH 7·0 and 8·0 and continued to increase above pH 8·0. At values above pH 8·0, interpretation became progressively more difficult owing to the reduction of viscosity of the substrate which occurred even in the absence of the enzyme. The pH optimum was therefore not determined precisely; it was, however, clearly at pH 8·0 or above. Tests of activity were generally made at pH 8·0, which was probably not far removed from the optimum and at which de-esterification and degradation by OH ions was relatively slow.

(*c*) *Effect of phosphate.* The effect of different concentrations of a Na_2HPO_4—KH_2PO_4 buffer at pH 8·0 added to pectin solutions adjusted to pH 8·0 were studied. Some reduction of activity was observed at a concentration of 0·012 M and this effect increased steadily with concentration until at 0·1 M only some 5 per cent. of the original activity remained. Relatively high concentrations were required therefore for any pronounced reduction of activity. As the concentration of phosphate in the culture filtrate could not have been higher than 0·007 M the substantial increase in activity following dialysis could not be attributed to removal of phosphate.

In general terms, therefore, the macerating and viscosity-reducing enzymes responded similarly to pH, phosphate and to high temperatures. But dialysis increased the activity of the viscosity-reducing enzyme much more than that of the macerating enzyme.

VI. Other Properties of Enzyme Solutions

(*a*) *Different pectic substances.* The effect of the standard enzyme solution on the viscosity of 0·5 per cent. solutions of different pectic substances was investigated. In the first series of experiments the solutions used were of (*a*) pectin, (*b*) sodium pectate obtained by neutralizing pectic acid with NaOH, and (*c*) sodium polypectate. The loss of viscosity, as a percentage of the total possible, was measured at intervals. On this basis sodium pectate solutions showed the lowest and sodium polypectate solutions the highest rate of viscosity loss, pectin solutions being intermediate.

Solutions of sodium pectate derived from pectin by de-esterification with NaOH at pH 10·0 or with tobacco leaf pectin-esterase at pH 7·0 behaved as did the solution prepared from pectic acid.

(*b*) *Measurement of activity by reducing group determinations.* Fission of the

1·4 glycosidic linkages between adjacent anhydrogalacturonic acid residues liberates reducing groups and their estimation has formed a basis for the determination of activity of polygalacturonase (Jansen and MacDonnell, 1945), it being understood that this enzyme specifically catalyses hydrolysis of this linkage. Filtrates from *F. moniliforme* cultures contain a fairly active polygalacturonase which may, of course, be identical with the viscosity-reducing enzyme dealt with above. The polygalacturonase activity of filtrates was studied with solutions of pectin and of sodium pectate derived from the pectin by enzyme de-esterification. A dialysed enzyme solution was used, as the undialysed filtrate contained calcium which formed a precipitate with solutions of sodium pectate. The final mixture in each case contained 30 per cent. by volume of the standard enzyme solution which, undiluted, macerated

TABLE III

Reducing Groups Liberated from Solutions of Pectin and Sodium Pectate

	mg. galacturonic acid			
	Pectin		Na pectate	
Sample	Exp. 1	Exp. 2	Exp. 1	Exp. 2
After 0·5 hr.	0	0	0	0
„ 1 „	0·1	—	0·19	—
„ 2 hrs.	0·1	—	2·03	—
„ 4 „	0·1	—	2·13	2·23
„ 8 „	0·1	0·1	2·71	2·91
„ 24 „	0·97	1·26	4·07	4·56
„ 48 „	1·94	2·43	4·37	4·95
„ 96 „	3·29	3·59	5·24	6·11
*„ 24+24 „	2·32	—	5·12	—

* Further enzyme added after 24 hours, and mixture incubated for another 24 hours.

potato tuber slices in 15–20 minutes. The enzyme/substrate mixtures were incubated at 25° C. under toluene; samples were removed at the intervals given in Table III, and their reducing power estimated by a modified Willstätter-Schüdel hypoiodite method (Jansen and MacDonnell, 1945). The results of two experiments with different enzyme preparations are given in Table III, in which the reducing power of the samples is expressed in terms of galacturonic acid.

The results from the two experiments agreed fairly well and showed the following: (i) the enzyme acts much more rapidly on solutions of sodium pectate than on pectin solutions, particularly in the earlier stages of the reaction, e.g. 8-hour samples from the sodium pectate mixtures had almost thirty times the reducing power of corresponding samples from the pectin mixtures. After 24 hours, however, the ratio was reduced to four and after 96 hours was less than two: (ii) some inactivation of the enzyme seemed to occur after 24 hours, since the addition of further enzyme caused a larger increase in reducing power: (iii) after 96 hours, the solutions of pectin and sodium pectate gave reducing group values corresponding to approximately 30 and 60 per cent.

respectively of the theoretical figure if complete hydrolysis to galacturonic acid had occurred.

The greater activity with sodium pectate as substrate is of interest in view of the fact that the standard enzyme solution produced a greater relative loss of viscosity of pectin than of sodium pectate solutions.

(*c*) *Chromatographic examination of breakdown products.* The samples described under (*b*) above were also used for analysis by paper chromatography, using Whatman No. 1 paper and the following solvent mixture—butanol 4 volumes, glacial acetic acid 1 volume, and water 5 volumes. The chromatogram was developed for 48 hours at room temperature (*c.* 20° C.) and sprayed with a reagent consisting of benzidine 0·5 g., glacial acetic acid 10 ml., 45 per cent. trichloroacetic acid 10 ml., and 95 per cent. ethanol 100 ml. The results clearly indicated that the action of the enzyme solutions on pectin and sodium pectate solutions was quite different although the latter were derived directly from the former by enzyme de-esterification, a mode of preparation which, in terms of viscosity, involved little degradation. In samples from the pectin mixtures, a trace of galacturonic acid appeared after 8 hours and the intensity of the spot increased with the age of the sample; no intermediate compounds were detected on the chromatogram. In the sodium pectate samples, traces of a component of higher molecular weight than galacturonic acid appeared after 4 hours. Thereafter, a series of components appeared; galacturonic acid was detected after 48 hours and increased in intensity in later samples. At least three components appeared between galacturonic acid and the base line. Only one of these persisted in the sample which received the most extensive enzyme treatment.

These results, which must be regarded as preliminary, suggest that the enzymes secreted by *F. moniliforme* act differently upon the esterified and de-esterified polygalacturonic acid chain. The esterified molecule of pectin may be broken down by the release of relatively small units, possibly galacturonic acid, to cause a gradual shortening of the chain and a gradual increase in the concentration of galacturonic acid with no or only a transient formation of higher molecular weight compounds. When sodium pectate is the substrate much larger units may be split off initially, galacturonic acid being formed only at later stages of the reaction. These effects could also be produced by different enzymes.

(*d*) *Comparison between enzyme preparations of* F. moniliforme *and other organisms.* A cell-free solution from cultures of *Bacterium aroideae* was diluted to give solutions which macerated potato tuber slices in 10–15, 30–35, and 55–60 minutes at pH 8·0, which is near the optimum value for activity. A preparation of *F. moniliforme* was similarly treated. These solutions were then added to the standard pectin substrate at pH 8·0 and the rate of viscosity loss determined. Some of the data obtained are given in Table IV, which expresses the loss of viscosity after 5 and 25 minutes as a percentage of the total possible. At each dilution, *B. aroideae* preparations caused a much greater loss of activity both after 5 and 25 minutes.

The viscosity/time curves obtained when pectin solutions are treated with *F. moniliforme* preparations were in fact quite different from those obtained with preparations from *B. aroideae* or from *Botrytis cinerea*, *Pythium debaryanum*, *Verticillium dahliae*, and *Sclerotium rolfsii*. When 10 per cent. by volume of preparations from these organisms, which macerated potato tuber slices in 10–15 minutes, were added to a 0·5 per cent. pectin solution, a very rapid drop in viscosity occurred within a few minutes; thereafter the fall in viscosity was much slower, but even so, within 20–30 minutes, the viscosity of the mixture was close to that of water. The sudden initial drop in viscosity did not occur when *F. moniliforme* preparations of similar macerating activity were used;

TABLE IV

Loss of Viscosity caused by Bacterium aroideae *and* F. moniliforme

Macerating activity (R.T. min.)	% loss in viscosity			
	B. aroideae		*F. moniliforme*	
	5 min.	25 min.	5 min.	25 min.
10–15	68	80	30	48
30–35	36	63	18	34
55–60	11	36	10	20

the loss of viscosity was gradual and even after 20–30 minutes' incubation a substantial part of the original viscosity of the mixture remained. The mode of action of *F. moniliforme* preparations would seem to be different, therefore, from that of the other organisms mentioned; further analysis of these differences is obviously required.

VII. Pathogenicity Tests

Apple and tomato fruit, narcissus and onion bulbs, potato tubers and turnip and swede roots were used in pathogenicity tests. The tissue was surface sterilized in each case and a plug measuring 3 by 5 mm. diameter was removed with a sterile cork-borer; the inoculum, consisting of a disc taken from the edge of an actively growing culture of *F. moniliforme* on potato-extract agar, was placed in the cavity, the plug replaced, and the broken surface sealed with a mixture of wax and 'Vaseline'. After inoculation, the material was kept in sterile tins at 25° C. for 2 weeks. Only apple and tomato fruit were attacked, 7 and 14 per cent. respectively of the fruit being rotted.

Pretreatment of tissues for 1–2 weeks at 5° or 35° C., increasing the water content of the tissues, or adding 1 per cent. solutions of glucose or pectin or the standard medium for protopectinase production to the inoculum, had little effect on pathogenicity.

The inability of the fungus to parasitize potato tubers was surprising in view of the suitability of potato extract for the secretion of macerating enzymes. In contrast, an isolate of *Fusarium avenaceum*, which was a much more active

pathogen of potato tubers, produced solutions having very little or no macerating activity when cultured on potato extract or on the standard medium used for *F. moniliforme* preparations. It is to be noted, too, that *F. moniliforme* caused some rot of apple tissue although apple extract was quite unsuitable for protopectinase secretion *in vitro*. These preliminary results clearly indicate a lack of correlation between pathogenicity and enzyme secretion *in vitro*. Similar results have been obtained with other host/parasite combinations, e.g. various bacteria and potato tubers (Lapwood, 1953), *Rhizopus* spp. and tubers of sweet potato (Harter and Weimer, 1921).

Discussion

The secretion of macerating enzymes in synthetic media by *F. moniliforme* was adaptive in that it occurred only when the media contained pectic substances or their degradation products. The suitability of certain natural products for enzyme production presumably depended, in part at least, on their content of these substances. The adaptive formation of the enzymes which degrade pectic substances *in vitro* remains to be investigated, but the fact that macerating enzymes were produced in response to reasonably well-defined pectic substances provides circumstantial evidence that maceration does involve the breakdown of similar compounds in plant tissue. The stimulation of enzyme secretion by galacturonic acid, the unit basis of the polygalacturonic acid chain believed to be the main constituent of pectic substances, was perhaps surprising, but similar results were obtained by Phaff (1947) in his work with *Penicillium chrysogenum*. It was also surprising to find that culture filtrates had little pectinesterase activity, since a number of other fungi produce this enzyme adaptively.

The experiments with natural extracts showed that there may be poor correlation between growth of the fungus and enzyme secretion. A number of extracts supported good growth but gave filtrates of low macerating activity. Apple extract was particularly striking in this respect. Not only did it fail to give active filtrates but, when added to a synthetic medium which by itself was suitable for enzyme secretion, greatly reduced the activity of the culture filtrates. This result was unexpected because the apple extract could be expected to contain relatively large quantities of pectic substances and because apples were parasitized by the fungus. The unsuitability of apple extract was not investigated further, but it seems likely that it contains one or more substances which inhibit the action of protopectinase. Other workers have provided evidence for the existence of such inhibitors; Brown (1915) found that an extract from broad-bean leaves was particularly inhibitory, while more recently Weurman (1953) has reported the existence of a 'pectinase' inhibitor in pears, and Cole (1953) has shown that oxidized apple and potato juice considerably reduced the activity of protopectinase and the viscosity-reducing enzyme contained in filtrates from cultures of *B. cinerea*. The presence of inhibitors may well account for the resistance of certain tissues to attack by some soft-rotting fungi, e.g. potato tuber, which is not

readily attacked by *B. cinerea* which nevertheless attacks a wide variety of other plant tissues.

The results of the pathogenicity tests make it clear that there may be no relation between the ability of an organism to secrete macerating enzymes in a tissue extract and to parasitize the tissue. Thus, *F. moniliforme* rotted apple fruit but did not produce any detectable macerating enzymes when grown in apple extract; in contrast, it produced these enzymes freely on potato extract but did not attack potato tubers.

In considering the properties of the pectic enzymes secreted by *F. moniliforme*, it must be stressed that maceration by filtrates from this, as from other micro-organisms, may involve the activity of more than one pectic enzyme and of enzymes attacking substrates other than pectic substances, e.g. cellulases and hemicellulases. Pectinesterase, however, seems to play no part in the process, as solutions of high macerating activity had negligible pectinesterase activity. The macerating enzymes have a high pH optimum and it may be significant that the fungus always caused a pronounced alkaline drift in media in which macerating enzymes were actively secreted. The viscosity-reducing enzyme also had a high pH optimum; in this respect and in its reaction to high temperatures and to phosphate, its properties resemble those of the macerating enzyme, but the enzymes responsible for the two types of activities behaved differently after dialysis, which considerably increased the activity only of the viscosity-reducing enzyme.

The fungus also secreted a polygalacturonase which resembled similar enzymes from other sources (Jansen and MacDonnell, 1945) in being more active with sodium pectate than with pectin as a substrate. Other properties of this enzyme were not investigated directly, but examination by chromatography of the products arising from the degradation of these substrates gave interesting results. Although the sodium pectate used was derived directly from the pectin by de-esterification with tobacco leaf pectinesterase which, presumably, did not affect the main structure of the polygalacturonic acid chain, the course of breakdown of the two substances was quite different. The degradation of sodium pectate followed the pattern which has been described by Jermyn and Tomkins (1950), i.e. a series of intermediate products and, finally, galacturonic acid were produced. The method of pectin breakdown has not been previously reported and needs further study. But the results so far obtained suggest that it may take place by the successive fission from the ends of the chain of units which may be as small as galacturonic acid itself. This would mean that the chain length would be reduced gradually and would possibly explain, in part at least, why *F. moniliforme* preparations reduce the viscosity of pectin solutions much more slowly than do preparations from other organisms which have a similar activity on tissue slices. There is therefore some evidence that *F. moniliforme* produces more than one enzyme able to attack pectic substances *in vitro*. The relation between these enzymes, if more than one exists, and protopectinase must await further investigation. It is, however, interesting to note that Ayres *et al.* (1952) have obtained evidence that

Aspergillus foetidus produces a number of pectic enzymes attacking the polygalacturonic acid chain of pectic substances.

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Studies on the Germination of Cereals

I. The Germination of Wheat Grains in the Ear during Development, Ripening, and After-ripening

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With eight Figures in the Text

ABSTRACT

The ability of individual grains to germinate in the ears of a red and a white wheat variety has been determined at different periods after anthesis, and at different moisture contents, before the stage of full maturity.

No grains germinated while active growth was taking place, but after desiccation during ripening, 88·5 per cent. of the white grains and 7 per cent. of the red grains were able to germinate in the ear; the percentage germination of the red grains increased to 83 per cent., when further desiccation occurred during the first 5 weeks of after-ripening, but some grains in the basal spikelets of the ears of both varieties failed to germinate until they had been subjected to the same desiccation from 13 to 23 weeks after anthesis.

The ability of the grains to germinate has been correlated with their desiccation at different stages during maturation, and the effect of certain factors, which inhibit the germination of immature grains, are discussed in relation to varietal differences in the colour of the grains and their position in the ear.

INTRODUCTION

THE ability of many seeds to germinate is influenced by their maturity and the conditions during development and ripening. In the case of the cereals, some grains may, if the weather is wet, germinate in the ear before they are sufficiently ripe for harvest, while others remain dormant after they have been harvested unless they have undergone a further process termed after-ripening. Many aspects of development in the cereal grain have been studied in detail, in particular the differentiation and growth of the embryo (Percival, 1921; Nutman, 1939, 1941), the accumulation of reserve materials (Brenchley, 1909; Porter, *et al.* 1950), and the changes which occur during germination (Brown and Morris, 1890; Brown, 1943, *a* and *b*). But no similar study has been made of the process of maturation whereby the grain acquires the ability to germinate, or of the factors which determine full maturity in this respect.

The complex process of maturation commences with the development of the embryo after fertilization; this occurs rapidly, and it has been shown that grains are able to germinate if harvested 5 days after anthesis and allowed to

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dry *in situ* on the straw (Harlan and Pope, 1922; Nutman, 1941). Growth of the pericarp and testa takes place in the early stages and is followed by the accumulation of starch in the endosperm, which, it has been suggested, induces a resting condition in the embryo (Pope, 1949). Progressive desiccation occurs during ripening and the moisture content of the grain reaches equilibrium with the atmospheric humidity when the supply of water from the parent plant ceases before harvest (Harlan and Pope, 1923). At this stage, which may be referred to as 'harvest-ripeness', some grains are fully mature and will germinate under favourable conditions while others require a period of after-ripening, of which the duration depends on the external conditions, before maturity is attained.

Observations on the germination of wheat grains in the ear have shown that varieties with white grains are more liable to sprout before harvest than those with red, although there are differences between individual varieties in each category (Harrington, 1932; Harrington and Knowles, 1940). Nilsson-Ehle (1914) suggested that these differences were due to hereditary factors, which determined the colour of the testa, as well as to other factors which controlled the rate of maturation. These factors still require analysis and it has yet to be established whether after-ripening constitutes a separate process in maturation. It may be due to a continuation of the process of desiccation which takes place before harvest, and the differences in the ability of grains to germinate may arise either from their different requirements for desiccation, or from differences in the time required for its effect to be manifest.

These possibilities have been examined in a comparative study of germination in the ears of a white-grained, and a red-grained, variety of wheat, carried out at different periods after anthesis corresponding to the stages of development, ripening, and after-ripening of the grains. For this purpose plants were grown under varying cultural conditions and, after harvest, ears were stored for different periods in atmospheres of controlled humidity, so that the relationship between grain moisture content and ability to germinate could be examined during ripening and after-ripening. The effect of varietal differences between the grains and their position in the ear was also investigated before and after harvest.

Experimental Procedure

The two wheat varieties used were *Triticum vulgare* var. Holdfast with white grains and *Triticum vulgare* var. Atle with red. They were known to resemble each other closely in habit of growth, size, and shape of the ear, as well as in the date of anthesis when sown in October.

The stage of development of the ear was defined in terms of the time elapsing from the start of anthesis, which had been determined by examining the ears daily after emergence and marking them with a tag as soon as anthesis was observed. At the time of each germination test the ripeness of the grains and their condition during after-ripening was determined on duplicate

samples of similar ears and characterized by their moisture content expressed as a percentage of the dry weight.

The method of inducing germination has already been described (Wellington, 1954). In outline the procedure was to soak the ears first for 8 hours in tap water through which air was bubbled continuously and then to place them in upright test-tubes in which they were incubated in a saturated atmosphere at 16° C. for a further 112 hours, thus making a total test period of 5 days. A sample of five ears taken at random from those which had started anthesis on the same day was used in each test and provided at least 200 grains. The variation between the percentage germination of individual ears was considerable when the mean of the sample approached 50 per cent., but the behaviour of the two varieties was so different that when the germination of one had reached this level, the germination of the other was either very low or very high, when the differences from the mean were small. The data relating to the grains in individual spikelets has been condensed by considering the two sides of the ear independently and numbering the spikelets in succession from the base on each side.

TABLE I

The Effect of Spacing and Anthesis Date on the Number of Grains in an Ear (mean of 50 ears)

Spacing	Anthesis date	Variety	
		White	Red
Normal	June 16	47·0	48·1
Wide	June 16	67·4	77·0
Wide	June 21	42·8	40·1

Conditions during ear development were varied by growing the plants at different spacings which, as shown by Engledow and Ramiah (1930), determines the number of ears on the plant, as well as the number of grains in the ear. It was thus possible to select, in each variety, ears with the same anthesis date containing different numbers of grains, as well as ears with approximately the same number of grains differing by one week in anthesis date.

Conditions during after-ripening were varied by storing replicate samples of ears, which had started anthesis and reached the stage of harvest ripeness at the same time, for varying periods in atmospheres of different relative humidity.

Ear development. The plants were grown at the normal spacing of 3 in., and at a wide spacing of 9 in. At the normal spacing each plant produced 1–3 ears with about 10 spikelets on each side and 2–3 grains in each spikelet. The plants at the wide spacing each produced 5–7 ears, the earlier ears having about 12 spikelets on each side of the ear and 2–4 grains in each spikelet, while the later ears resembled those from the close spacing. The number of grains in the ears from the normal spacing, and in the late ears from the wide spacing, was approximately the same in both varieties, but there were more grains in the early ears of the red variety than in those of the white. The effect of spacing and anthesis date on the number of grains in an ear is shown in Table I.

Anthesis. In both varieties anthesis started first in the ear on the main shoot and then in succession in the ears on shoots of higher order. The percentage of spikelets which started to anthese during the first 24 hours, at different positions in the ear, is shown in Table II for the ears from the normal spacing; a similar distribution was found in the other ears and there did not appear to be any difference between the two varieties. The middle spikelets were the first to start anthesis followed next by those at the top of the ear and finally by those at the base. This is in agreement with the earlier observations of Percival (1921), who found that anthesis was delayed in the basal spikelets by 2–4 days. The flowers in each spikelet anthesed in succession, starting at the

TABLE II

The Proportion of Spikelets showing Anthesis after 24 hours in Ears at the Normal Spacing (percentage of 40 spikelets)

Spikelet number	Variety	
	White	Red
11	5·0	0
10	7·5	2·5
9	22·5	10·0
8	42·5	37·5
7	65·0	70·0
6	72·5	85·0
5	50·0	72·5
4	15·0	47·5
3	0	10·0
2	0	0
1	0	0

lowest, but the distribution of anthesis in the ear soon became complex because some of the upper flowers in the top spikelets anthesed before the lower flowers in the basal spikelets. The time required for the completion of anthesis in any one ear was estimated at 5–7 days, but depended greatly on the weather; Percival recorded 3–5 days in fine weather and 6–8 days when it was wet and dull.

EXPERIMENTAL RESULTS

Changes in moisture content during ripening and after-ripening

The changes in the percentage moisture content which took place during the development and ripening of the grain are shown in Fig. 1, for the whole grain and for the embryo in both varieties. There was a progressive reduction in the percentage moisture content of the whole grains between the second and the eighth week after anthesis, when equilibrium was reached with the atmospheric humidity at a value of 19 per cent. (dry weight) and the grains were considered to be harvest ripe. The percentage moisture content of the embryo fell at a similar rate to the rest of the grain between the second and fifth week after anthesis, but no further reduction occurred and the percentage moisture content remained at 120 per cent. (dry weight) until the seventh

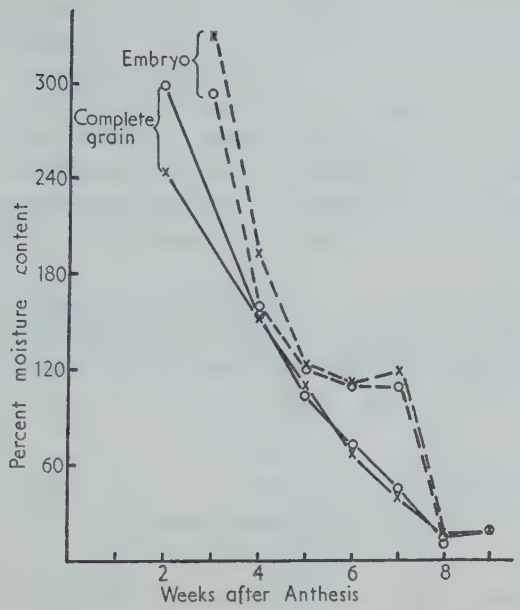

FIG. 1. The percentage moisture content of the complete grain and the embryo, 2–9 weeks after anthesis. White wheat (O), Red wheat (X).

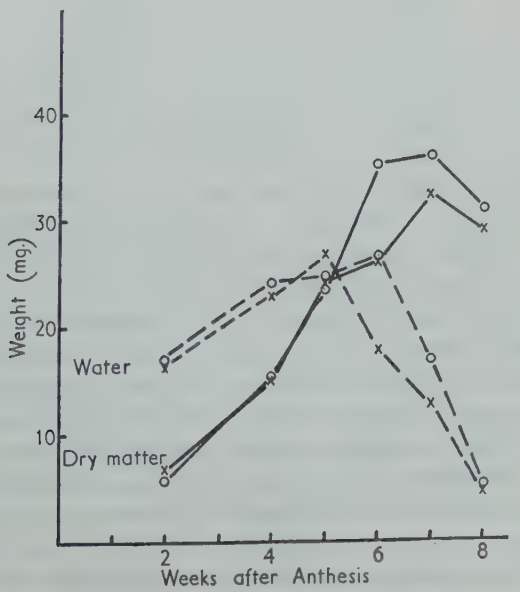

FIG. 2. The dry weight and water content of the complete grain, 2–8 weeks after anthesis, White wheat (O), Red wheat (X).

week, when there was a final rapid reduction to the same level as the rest of the grain at the harvest ripe stage. It was observed that the covering layers over the endosperm started to lose their green colour during the sixth week after anthesis, although this did not occur in the region of the embryo until the eighth week. The change in colour was due to the disintegration of the chloroplasts in the cross layer of the pericarp and was followed by the development of a pigment in the testa of the red grains but not in the white grains, which remained light brown in colour. The time when this change occurred appeared to be related to the moisture content of the tissues beneath the covering layers.

It will be seen from the changes in the actual water content and dry weight of the grains, which are shown in Fig. 2, that the desiccation of the grain between anthesis and the harvest ripe stage took place in three stages; these are similar to the stages which Harlan and Pope (1923) have described in the barley grain.

TABLE III

The Effect of Spacing and Date of Anthesis on the Percentage Moisture Content of Grains during Ripening

Weeks from anthesis	Variety	Normal spacing	Wide spacing	
			Early anthesis	Late anthesis
5	White	106	103	97·9
	Red	111	102	102
6	White	73·0	78·5	75·8
	Red	67·7	76·2	77·1
7	White	47·0	56·3	29·6
	Red	39·6	57·8	31·4
8	White	16·5	17·7	18·3
	Red	16·5	18·7	18·3

During the first stage, which lasted until the fifth or sixth week depending on the variety, there was an increase in both the water content and the dry weight, but as the rate of increase was greater in the case of the latter this resulted in a reduction in percentage moisture content as is shown in Fig. 1. The dry weight continued to increase during the second stage, which lasted from the fifth to the seventh week after anthesis, when starch was accumulating in the endosperm, but the water content fell, and the effects of desiccation first became apparent with the change in colour of the covering layers. The third stage took place during the eighth week after anthesis, after the grain had reached its maximum dry weight, when the moisture content fell rapidly. This caused a reduction in its volume as the endosperm became hard, and the covering layers assumed their characteristic wrinkled appearance.

The effect of spacing and anthesis date on the changes in moisture content of the grain during ripening can be seen in Table III. Although there was no effect during the initial stages of development, during the seventh week after anthesis the moisture content of the grains in the late ears from the wide

spacing was lower than in the early ears, with the moisture content of grains in the ears from the normal spacing intermediate between the two. At the same time a marked gradient also developed in the moisture content of the grains in successive spikelets in the ear, which is shown in Table IV for both varieties. The grains in the top spikelets lost water more rapidly than the rest, and their moisture content was 20–25 per cent. lower than that of the grains in the basal spikelets. The covering layers of the top grains were also the first to lose their green colour, which they did a week before those at the base of the ear.

TABLE IV

The Effect of the Position of the Spikelet in the Ear on the Moisture Content of the Grain at 7 weeks after Anthesis

Spikelet number	Variety	
	White	Red
12	31·5	38·3
11	35·8	41·7
10	34·8	41·5
9	37·8	42·8
8	42·8	46·5
7	46·0	48·7
6	52·8	50·8
5	50·4	50·6
4	54·1	56·0
3	58·9	55·9
2	57·5	55·6
1	66·2	57·8

Ears, harvested during the eighth week after anthesis when the grain moisture content had reached 19 per cent., were stored over mixtures of water and sulphuric acid to provide relative humidities of 50 and 75 per cent., in a cellar at a relative humidity of 85–90 per cent., and in a saturated atmosphere over water. The initial moisture content remained unchanged at 85–90 per cent. RH, except for a slight rise to 20 per cent. after 5 weeks storage. In the saturated atmosphere the moisture content continued to rise throughout the storage period and had increased to 30 per cent. after 2 weeks and 40 per cent. after 5 weeks; while the moisture content at 50 per cent. RH and 75 per cent. RH was reduced to 10 per cent. and 14 per cent. respectively during the first 2 weeks of storage and thereafter remained at these levels.

Germination during ripening. In each variety three series of ears were selected to include the two spacing treatments and two dates of anthesis at the wide spacing, and these were tested for germination at weekly intervals from the second week until the eighth week after anthesis. The results for the two varieties are shown in Fig. 3. No grains germinated at 5 weeks after anthesis in any ears, but at 6 weeks between 1 and 5 per cent. had germinated in all except the late ears of the white wheat, which attained a percentage germination of 29 per cent. There was a marked increase in the percentage germination of grains in the ears of the white wheat at 7 and 8 weeks after

anthesis, and at the harvest ripe stage the germination in all three series of ears was 80–90 per cent. During this period, however, comparable series of ears of the red variety showed only a slight increase in percentage germination, reaching 7–10 per cent. at the harvest ripe stage.

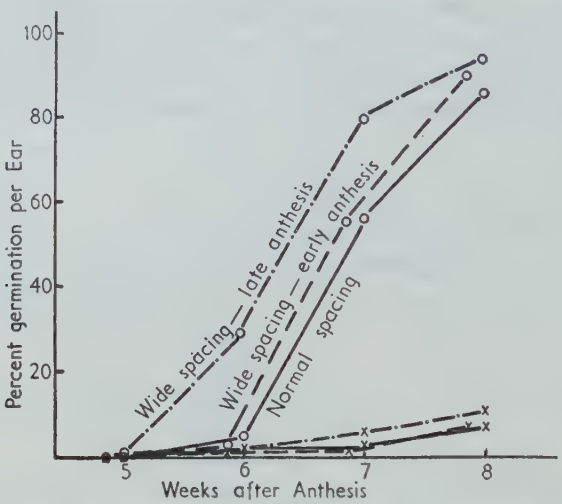

FIG. 3. The percentage germination in the ear, 5–8 weeks after anthesis. White wheat (O), Red wheat (X).

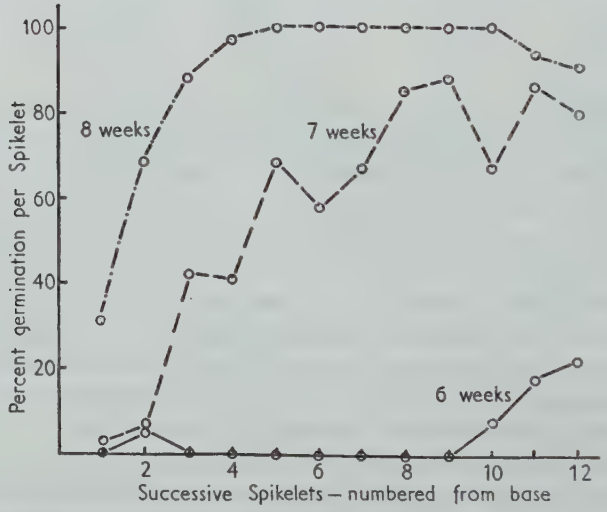

FIG. 4. The percentage germination in successive spikelets in ears of the white wheat, 5, 7, and 8 weeks after anthesis.

The percentage of grains capable of germinating at a given stage of development was independent of the number of grains in the ear so long as the ears had begun anthesis at the same time. However, a greater proportion of the white grains were able to germinate in the late ears, during the sixth and seventh weeks after anthesis, than in the early ears, and with this was associated

a greater number of grains in which the covering layers had changed colour; whereas the percentage germination in late and early ears of the red variety differed little.

The position in the ear also affected the time at which the white grains were first able to germinate, as is shown in Fig. 4 by the percentage germination in successive spikelets at 6, 7, and 8 weeks after anthesis. The first grains to germinate at 6 weeks were those at the top of the ear, which had started to change colour, and at 7 weeks there was a similar gradient in the percentage of grains which germinated in successive spikelets to that in the percentage moisture content already mentioned (Table IV). By the time that all the grains in the ear had reached the harvest ripe stage 90–100 per cent. of the white grains were capable of germinating in the fourth and higher spikelets, but only about 30 per cent. in those at the base. In the ears of the red variety, the number of grains which were capable of germinating was, however, too small to obtain an accurate percentage for the different spikelets at 6 and 7 weeks, but the distribution at 8 weeks and after which is shown in Fig. 6 presents a somewhat similar picture.

In both varieties it appeared that the grains were unable to germinate as long as the pericarp remained green, but as soon as the covering layers had changed colour a high proportion of the white grains in the middle and top spikelets were able to germinate. Very few of the grains in similar spikelets of the red variety were able to germinate, nor were many of the grains in the lowest spikelets in the ears of either variety.

Germination during after-ripening. At the harvest ripe stage the proportion of grains capable of germination was 88·5 per cent. in the ears of the white variety and 7 per cent. in the ears of the red variety; the results of further tests made at intervals during the next 5 weeks on ears stored at different relative humidities are shown in Fig. 5. The percentage germination of the red grains, stored at 85–90 per cent. RH had increased to 28 per cent. after 2 weeks and to 56 per cent. after 5 weeks; there was also a further increase of 5 per cent. in the percentage germination of the white grains stored for 5 weeks under the same conditions. The percentage germination of the red grains stored in a saturated atmosphere also increased to 40 per cent. after 5 weeks, although there was little increase during the first 2 weeks of storage. Storage under saturated conditions reduced the percentage germination of the white grains to 81·5 per cent., and it was found that in 78·3 per cent. of the grains which failed to germinate the embryos showed evidence of decay, whereas only 6·0 per cent. of the red grains were similarly affected. The distribution of the red grains, which germinated in the ears stored at 85–90 per cent. RH, is shown in Fig. 6 for 8, 10, and 13 weeks after anthesis. It will be seen that the gradient in the percentage germination in successive spikelets at 13 weeks was similar to that which was found in the white ears at 7 weeks after anthesis. Since the moisture content had not been reduced below the level at the harvest ripe stage, further desiccation during after-ripening was not essential for some of the red grains to acquire the ability to germinate.

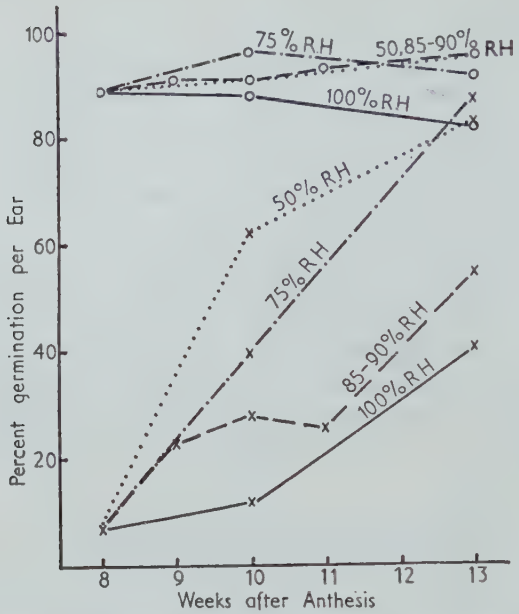

FIG. 5. The percentage germination in ears maintained at different relative humidities, 8–13 weeks after anthesis. White wheat (O), Red wheat (X).

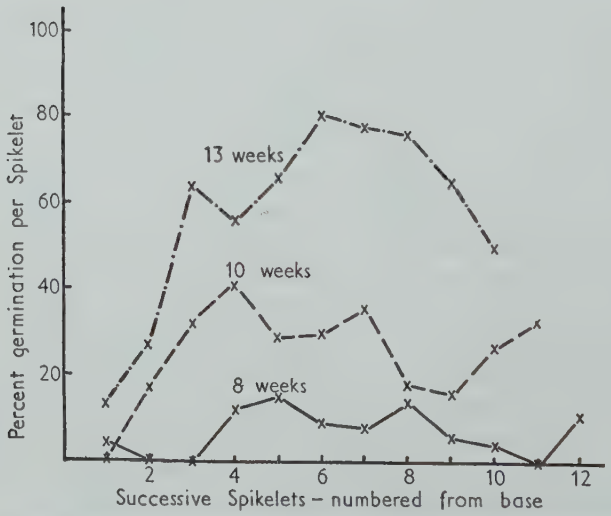

FIG. 6. The percentage germination in successive spikelets in ears of the red wheat, 8, 10, and 13 weeks after anthesis.

When the moisture content was reduced during storage to a level below that at the harvest ripe stage, there was, however, a marked increase in the percentage germination of these grains. After 2 weeks' storage in atmospheres of 50 per cent. RH and 75 per cent. RH, germination had increased to 62 per cent. and 39 per cent., respectively, and after 5 weeks' storage it reached 80–90 per cent. under both sets of conditions. By contrast there was no further increase in the percentage germination of the white grains under these conditions as compared with 85–90 per cent. RH.

Reduction in the moisture content of the grains after they had reached the harvest ripe stage, therefore not only increased the total number of red grains able to germinate after a given period but also increased their rate of maturation. As the storage period had been restricted to 5 weeks there was no

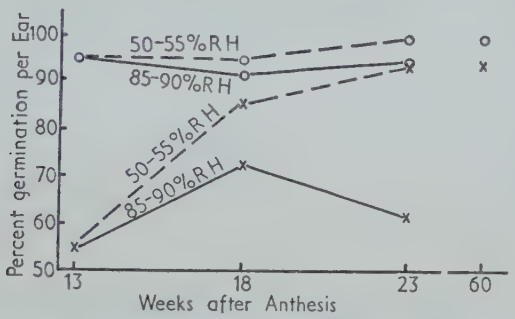

FIG. 7. The percentage germination in ears stored at different relative humidities from 13 weeks after anthesis. White wheat (O), Red wheat (X).

indication whether those grains which only germinated after further desiccation would have eventually done so without any reduction in moisture content. In both varieties there was also present a number of grains which did not acquire the ability to germinate during this period, even after additional desiccation.

A further investigation was carried out in which half of the ears, which had already been stored for 5 weeks at 85–90 per cent. RH, were then removed to a dry laboratory with a relative humidity of 50–55 per cent. RH. The results of the germination tests carried out on these ears as well as on those which remained at 85–90 per cent. RH, after further periods of 5 weeks and 10 weeks, are shown in Fig. 7. The percentage germination of the red grains in the ears which were stored under the dry conditions had increased to 95 per cent. at 23 weeks after anthesis, which was the same value as that obtained with ears containing fully mature grains which had been kept for a year in dry storage. The percentage germination in the ears which were stored at 85–90 per cent. RH increased to 72 per cent. after 5 weeks' storage, but then fell to 61·5 per cent., and as the embryos in the ungerminated grains showed no visible sign of decay, it is possible that they had developed a secondary dormancy similar to that described in mustard by Kidd and West (1917). After 10 weeks' dry storage the percentage germination of the white

grains had meanwhile increased to 100 per cent. while that of the grains kept at 85–90 per cent. RH remained at 95 per cent.

The distribution of germinated grains in the individual spikelets of both varieties, after further storage at 50–55 per cent. RH and 85–90 per cent. RH, is shown in Fig. 8; it will be seen that in both cases the grains in the basal spikelets acquired the ability to germinate in dry storage but not when their moisture content remained at the same level as at the harvest ripe stage.

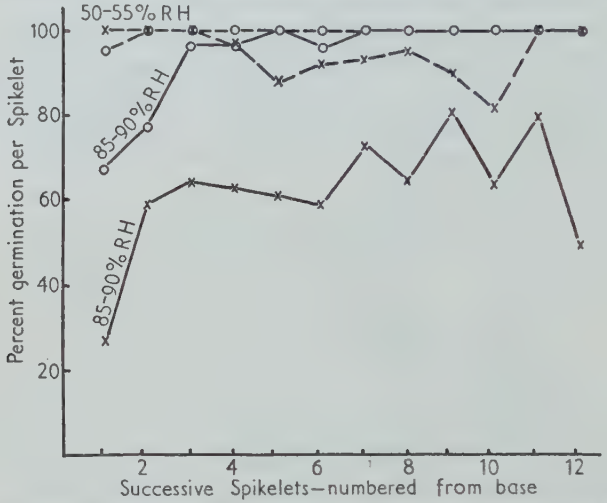

Fig. 8. The percentage germination in successive spikelets at 23 weeks after anthesis. White wheat (O), Red wheat (X).

Discussion

In the ears of both varieties the onset of anthesis results in a complex relationship between the individual grains owing to the different times at which maturation starts. This relationship is further complicated during ripening since the moisture content falls more rapidly in some grains than in others. The experimental results show, however, that all of the grains in the ear reach maturity after they have been subjected to desiccation for certain periods after anthesis. It is also apparent that the requirement for desiccation at a particular stage of maturation depends on the variety and on the position of the grain in the ear.

The relation between the stage of maturation, the degree of desiccation, and the ability of the grains to germinate is evident from Table V, in which is summarized the data relating to the duration of each stage (in weeks after anthesis), as well as the percentage moisture content and percentage germination in the ear at the end of each stage.

Stage I. The first stage lasted from 2 to 5 weeks after anthesis during which the grains were growing rapidly, but in spite of this the percentage moisture content fell because the dry weight increased more rapidly than the water content. However, this desiccation was not accompanied by any visible

changes in the grains which in both varieties remained green and were incapable of germination.

Stage II. The second stage lasted from 5 to 8 weeks after anthesis when the grains reached their maximum dry weight and the changes characteristic of ripening occurred. The moisture content fell as a result of desiccation caused at first by the accumulation of starch in the endosperm, and then by the evaporation of water into the atmosphere until equilibrium was reached. At an early stage this was associated with the disintegration of the layer in the pericarp which contained chloroplasts and the disappearance of the chloro-

TABLE V

Stage of maturation	Duration (weeks after anthesis)	Desiccation (% moisture content)	Germination (% germinated grains per ear)	
			White	Red
I (Growth)	2–5	103	0	0
II (Ripening)	5–8	19	88·5	7
III (After-ripening)	8–13	19	95	55
		10	95	83
IV (After-ripening)	13–23	19	95	62
		10	100	95

phyll. The varietal difference in the colour of the grains then became apparent, as the pigment appeared in the testa of the red grains and the endosperm became hard. There was a rapid increase in the percentage of white grains, but not of the red grains, which were able to germinate as soon as the covering layers lost their green colour. The first of the white grains to germinate were those at the top of the ear, which were the first to change colour, and a germination gradient was established as the grains at the top of the ear lost moisture more rapidly than those at the base. The percentage germination in the late ears was higher than in the early ears, at a corresponding stage of development, and the grains in them also lost moisture more rapidly during ripening.

Stage III. The third stage lasted from 8 to 13 weeks after anthesis, when after-ripening occurred and further desiccation depended on a reduction in the relative humidity of the surrounding atmosphere. There was an increase in the percentage germination of the red grains without any further desiccation, and a germination gradient developed similar to that in the white ears at the preceding stage. The percentage germination increased more rapidly, however, when the moisture content was reduced. But few of the grains of either variety in the basal spikelets were capable of germinating at this stage.

Stage IV. The fourth stage lasted from 13 to 23 weeks after anthesis, when a similar reduction in the moisture content enabled all of the grains in the basal spikelets to acquire the ability to germinate, whereas, without this

additional desiccation, the percentage germination of the red grains first increased slightly, and then fell, while the germination of the white grains remained unchanged.

During the first stage of maturation, the presence in the pericarp of an intact layer containing chloroplasts appears to inhibit the germination of the grains of both varieties. A similar effect has been reported in the immature grains of rye by Hatcher (1945), and in the unripe seeds of mustard and peas by Kidd and West (1920), and is probably widespread. In wheat the effect disappears when the immature grains are harvested and dried first (Hatcher and Purvis, 1946); although it seems probable that as in barley (Harlan and Pope, 1922), and rye (Nutman, 1941), there is a period immediately after fertilization when germination will not occur even when the grains are dried. The degeneration of this layer in the pericarp accompanies the desiccation which occurs during the second stage of maturation, and enables a high proportion of the white grains to germinate; the majority of the red grains are, however, still prevented from germinating by a factor which appears to be associated with the development of red pigment in the testa, but which may also be due to a difference in its structure (Nilsson-Ehle, 1914). There is also a further factor, associated with the position of the grain in the ear, which in both varieties prevents the germination of grains in the basal spikelets at this stage. The 'varietal' factor slowly disappears with time, and is removed more rapidly by additional desiccation during the third stage of maturation, whereas the 'basal' factor is not eliminated by additional desiccation until the fourth stage of maturation.

Once the inhibiting effect of the green layer in the pericarp has been removed during ripening, the proportion of grains which are able to germinate in the ear is determined by the time after anthesis, and the desiccation which they have undergone. This is confirmed by the observation of Stapledon and Adams (1919) that the percentage germination of many wheat samples was increased after harvest if they were either dried at 40° C. for 3 days or stored in the laboratory for 2–3 weeks. The differences between the varieties appear to arise from the fact that a greater degree of desiccation is required to remove the inhibiting effect of the covering layers in the red grains than in the white; and in so far as the desiccation, which occurs after the grains have reached the harvest ripe stage, depends initially on the humidity of the atmosphere in the field, this will also determine the proportion of grains which are liable to sprout in the ear or require after-ripening. The differences in the behaviour of the grains of both varieties which arise from their position in the ear, indicate that desiccation is also likely to have a direct effect on the maturation of the embryo in addition to its effect on the covering layers. While these experiments give no indication of the nature of the factors which inhibit the germination of grains in the basal spikelets, they may be related to fundamental differences in development, because the flower primordia in this part of the ear are the last to be differentiated, the spikelets are the last to anthese, and the grains are the last to reach harvest ripeness.

Summary

1. A comparative study has been made of germination in the ears of a red and a white wheat variety during the development, ripening, and after-ripening of the grains.

2. No grains in either variety germinated so long as the layer in the pericarp containing chloroplasts remained intact.

3. When this layer disintegrated during ripening the majority of the white grains were able to germinate, but only a few of the red grains.

4. The proportion of red grains which were able to germinate increased after harvest without any further desiccation, but there was a greater increase when the moisture content of the grains was reduced.

5. In both varieties some of the grains in the basal spikelets of the ear were unable to germinate until they were subjected to further desiccation after a period of storage.

6. At a given stage in maturation the proportion of grains which will germinatein the ear is determined by the time after anthesis, and the desiccation which has taken place. And once the harvest ripe stage has been reached the latter is determined by the humidity of the atmosphere with which the grains have been in equilibrium.

7. The majority of the red grains have a greater requirement for desiccation than the white, but in both varieties the grains at the base of the ear have a special requirement for desiccation.

Acknowledgements

I wish to thank Professor F. G. Gregory, F.R.S., for much encouragement and stimulating discussion during the course of this investigation. I should also like to record my thanks to Mr. F. R. Horne and Mr. C. C. Brett for facilities without which it could not have been undertaken.

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Experimental and Analytical Studies of Pteridophytes

XXXII. Further Investigations on the Effect of Undercutting Fern Leaf Primordia

BY

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With Plate III and fifteen Figures in the Text

Abstract

An account is given of experiments in which young primordia and primordium sites at the apex of *Dryopteris aristata* were isolated basally and laterally by undercutting and deep radial incisions. The treatments had the effect of destroying or severing the incipient vascular tissue underlying the primordium on its abaxial and lateral sides. Provided the shoot apex had not been damaged during treatment, neither buds nor centric (or radial) leaves were induced as a result of the experimental procedure employed. On the contrary leaves with distended bases, showing an abnormally rapid rate of growth, were usually produced. The results obtained are against the view that a leaf-trace, differentiating acropetally in advance, determines the formation of a primordium; but they support the thesis that the apical cell group exercises a regulative effect on the growth and morphogenetic activities of the apical meristem and young primordia. The evidence also supports the view that the incipient vascular tissue is important in morphogenesis in that it affords a pathway for the translocation of nutrients and hormonal substances.

Introduction

VARIOUS observers, e.g. Sterling (1945) in a study of the shoot apex of *Sequoia sempervirens*, have suggested that leaf-traces are formed in advance of the leaf primordia to which they relate; in other words, that an acropetally differentiating leaf-trace determines the position in which a leaf primordium will be formed. This idea has been tested experimentally by M. and R. Snow (1947) using *Lupinus*. They found that if the positions of the next primordia to be formed, i.e. I_1 and I_2, are undercut, leaf primordia are nevertheless formed in these positions. This finding has been supported in experimental investigations undertaken by Ball (1948), and by the writer (Wardlaw, 1949*b*) using *Dryopteris aristata*. When transverse incisions were made below the primordium sites I_1, I_2, and I_3, leaf primordia were subsequently formed. In other words, the severing of direct connexion with the incipient vascular tissue (or pre-vascular tissue) in the shoot did not prevent leaf inception. In recent attempts to throw new light on factors which may determine the characteristic orientation and dorsiventral symmetry of leaves in this fern, various surgical treatments of the shoot apex have been attempted. In some of these experiments, in which the aims were to interfere with the supply of nutrients to very young leaf primordia and primordium sites, and to

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modify the normal distribution of growth in characteristic ways, results of some interest have been obtained. These are briefly considered in the present paper.

Materials and Experimental Treatments

Large apices of *Dryopteris aristata* (*D. austriaca*) were laid bare as described in earlier papers in this series. The short pieces of shoot bearing these apices were planted in moist peat. Very fine knives were used for making the incisions. The terminology for leaf primordia and primordium sites is that now commonly used in studies of phyllotaxis.

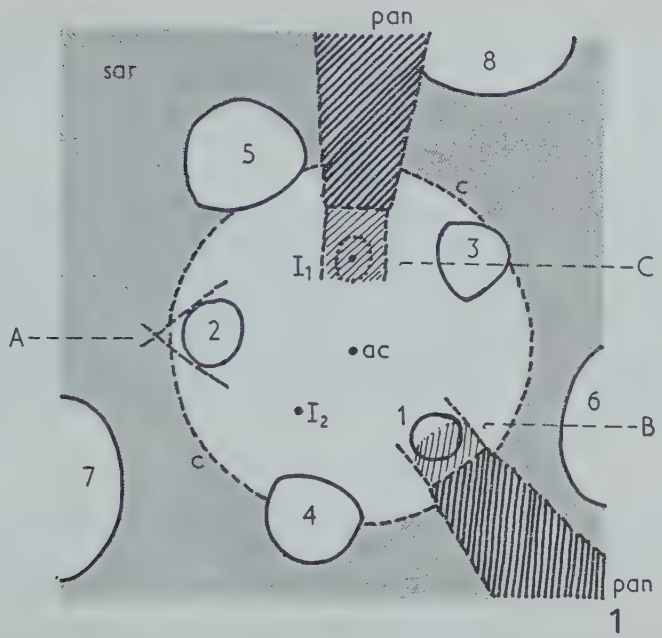
In the fern apex there is a steady increase in the rate of radial growth from the apical cell to the base of the cone and a rapid acceleration of this component of growth in the sub-apical region. A new leaf primordium, which is first apparent near the base of the apical cone as a low, circular or elliptical mound of meristematic cells, becomes extended tangentially and abaxially and the characteristic orientation and dorsiventral symmetry of the primordium are established. These tangential and basal enlargements of the primordium apparently stand in a direct relationship with the growth which is taking place in that part of the shoot apex. Further, in a large *Dryopteris* apex, the amount of vertically downward displacement of a new primordium from the apical cell during the initial plastochrones is small compared with the radial, and therefore with the tangential, components of growth of the axis. This being so, it seemed possible that if the tangential and basal growth of a primordium could be restricted, a lateral member of radial symmetry, i.e. a bud or a centric (or radial) leaf, might be obtained.

The treatments applied in the experiments to be described here are indicated diagrammatically in Text-figs. 1 and 2, and were as follows:

(*a*) The primordium or site was isolated basally and laterally by two downwardly converging deep cuts, made at right angles to the surface of the apex. The intention was to restrict the lateral and basal enlargement of the primordium, Text-fig. 1A.

(*b*) The abaxial side of a primordium or site was isolated about half-way up by two deep radial-longitudinal incisions and by undercutting, Text-fig. 1B. The intention was to restrict the access of nutrients to the primordium laterally and basally. As the path of nutrient supply would be, to some extent, deflected towards the adaxial side of the primordium, there was the possibility that growth on that side might be increased, with consequential effects on the morphological development of the primordium.

(*c*) The primordium or site was isolated on all but its adaxial side by being almost completely undercut and by two deep longitudinal radial incisions, Text-figs. 1C, 2. In some experiments the latter were omitted, as the delicate tissue did not stand up well to so much treatment. In these experiments the intentions were (i) to create a situation in which the path of nutrients to the primordium would be very largely by way of the adaxial side, and (ii) to eliminate the outward thrust by the radial component of growth.

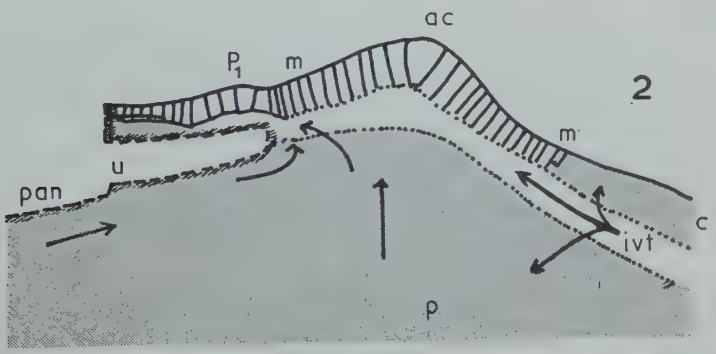


TEXT-FIG. 1. Diagrammatic representation of the apex of *Dryopteris* as seen from above, showing leaf primordia 1–8, the site of the next primordium to be formed, I_1, and the next after that, I_2; *ac*, position of apical cell; *cc*, the region of transition from the base of the apical cone to the sub-apical region, *sar*.

Experiment A. A primordium isolated laterally and basally by two deep, downwardly converging incisions, made at right-angles to the surface of the cone.

Experiment B. A primordium isolated by two deep radial incisions and by partial undercutting parallel to the surface of the cone.

Experiment C. The site of I_1, isolated by deep radial incisions, extending adaxially, and by extensive undercutting parallel to the surface of the cone. *pan*, panel of tissue removed to facilitate controlled undercutting. (×30.)



TEXT-FIG. 2. Longitudinal median section of an apex, passing through the youngest primordium, P_1, which has been deeply undercut, *u*; *pan*, position from which panel of tissue has been removed to facilitate undercutting; *ac*, apical cell of axis; *mm*, apical meristem; *ivt*, incipient vascular tissue; *p*, pith; *c*, cortex. The arrows suggest how nutrients may be distributed from the incipient vascular tissue (right), and how they may reach the laterally and basally isolated primordium (left). (Semi-diagrammatic, ×55.)

In the matter of technique, the procedure adopted in (*b*) and (*c*) above was as follows, Text-figs. 1, 2. A rectangular panel of tissue was first removed from the abaxial side of the primordium to admit of access for undercutting. A very small knife blade, with both of its edges and its tip ground very thin on a fine carborundum stone, could then be inserted under the primordium parallel to the surface of the apical cone. As the blade could be seen through the translucent tissue, the extent of the undercutting could be controlled. The incipient vascular tissue of the stem on the abaxial side of the primordium was thus removed, while that underlying the primordium was destroyed, according to the extent of the undercutting. As a precocious leaf-trace has not been observed in the I_1 site in *Dryopteris*, a deeply undercut I_1 site will have no underlying pre-vascular tissue; but a very small amount of pre-vascular leaf-trace tissue *might* remain associated with similarly undercut P_1 and P_2 primordia.

Observations

Except in those specimens in which the shoot apex had been damaged during treatment, or in the course of the experiment, buds were not induced as a result of any of the treatments and centric leaves were not obtained.

Experiment (*a*)*: Primordia or sites isolated laterally and basally*

Observations on 23 treated primordia and sites, including P_2, P_1, I_1, and I_2, in 10 intact apices, were recorded, Table I. In the course of 24 days no buds

Table I

Primordia treated		Leaves formed	Leaves not formed
I_2	2	1	1
I_1	9	5	4
P_1	10	8	2
P_2	2	1	1

were formed; most of the P_1's and P_2's developed into large primordia with distended parenchymatous bases; I_1 and I_2 sites yielded leaf primordia, some of which were small; some of these sites grew into scaly parenchymatous mounds and some became necrotic. New primordia, up to I_6 in one apex, were formed in the normal sequence.

Experiment (*b*)*: Primordia or sites partially isolated abaxially by undercutting and by radial incisions*

Observations on 21 primordia, including I_2–P_2, in 11 intact apices, were recorded, Table II. In the course of 3–4 weeks no buds were obtained;

Table II

Primordia treated		Leaves formed	Leaves not formed
I_2	1	1	—
I_1	8	8	—
P_1	9	8	1
P_2	3	3	—

20 of the treated primordia and sites developed as leaves, usually with conspicuously swollen bases; new primordia, up to I_6, developed in the normal phyllotactic sequence in some apices.

Experiment (c): Primordia or sites isolated by deep undercutting, without radial incisions

Observations on 13 treated primordia, including I_1–P_2, in 5 intact apices, were recorded, Table III. In the course of 3–4 weeks no buds were obtained; 10 primordia developed as leaves, mostly with conspicuously swollen bases; two I_1 sites each yielded a small leaf primordium subtended by a conspicuous parenchymatous mound; three I_1 sites developed as swollen, scaly mounds without primordium formation; other new primordia developed as in experiments (*a*) and (*b*).

TABLE III

Primordia treated		Leaves formed	Mounds formed
I_1	5	2	3
P_1	5	5	—
P_2	3	3	—

Where extensive undercutting was accompanied by deep radial incisions, many of the treated primordia or sites became necrotic. The primordia that survived the treatment had typically distended basal regions as described above; no buds were obtained.

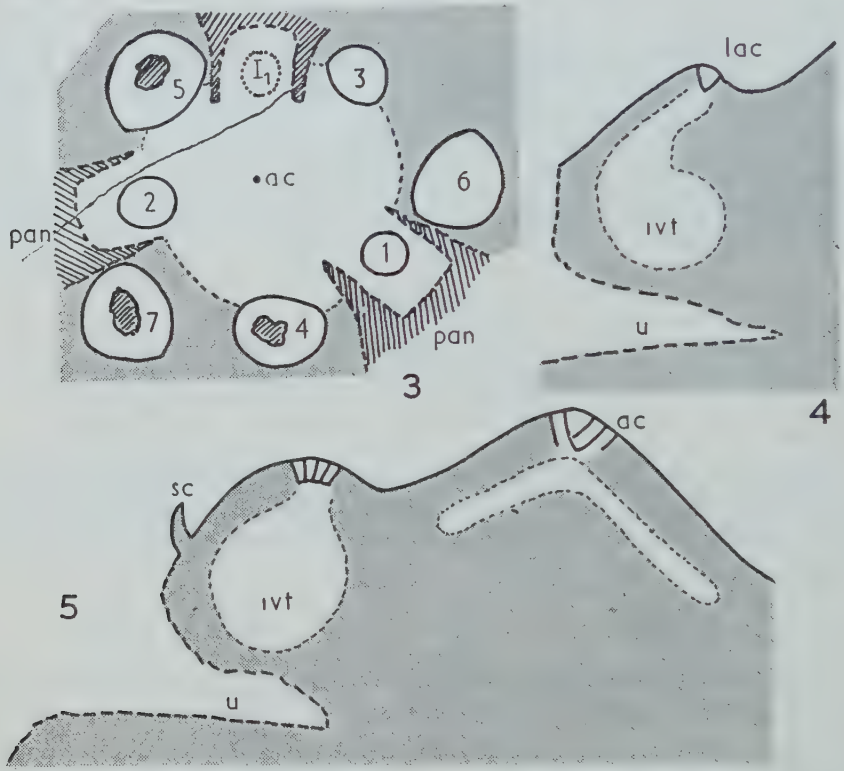
Anatomical observations

The more interesting specimens from these experiments were fixed and cut as longitudinal or transverse serial sections.

In Experiment (*a*) the enlarged leaf primordia were like those already described by the writer (Wardlaw, 1949*c*) when primordia were isolated by two deep radial incisions.

Text-fig. 3 illustrates an apex, 3 weeks after treatment, in which I_1, P_1, and P_2 had been partially isolated and undercut as in Experiment (*b*). Each treated primordium has a large, swollen basal region. Text-figs. 4 and 5 show the extent of the undercutting of I_1, its development as a leaf primordium with a large basal region, and its conspicuous vascular tissue. The undercutting incision which was originally approximately parallel to the surface of the cone is now horizontal, Pl. III, Fig. 2. Text-figs. 6 and 7 show another apex in which P_1 had been partially undercut, Experiment (*b*). P_1 has developed to very large size as compared with P_2 or I_1. In longitudinal sections, Text-fig. 7, the conspicuous vascular tissue of this primordium was seen to have no basal contact with that of the axis, Pl. III, Fig. 1; but in such specimens, especially where the radial cuts were inextensive acropetally, there was median-lateral continuity of leaf-trace and axial vascular tissue, Text-figs. 10, 11. In Text-fig. 8, also illustrating Experiment (*b*), I_1 and P_1, which

had been partially isolated, have conspicuously distended basal regions. The serial transverse sections, Text-figs. 9–11, show the large relative development of the leaf primordium in the I_1 site, with its conspicuous vascular tissue, Text-fig. 9. This vascular tissue is continuous with that of the axis in what may be described as a median-lateral position, Text-fig. 10, but below

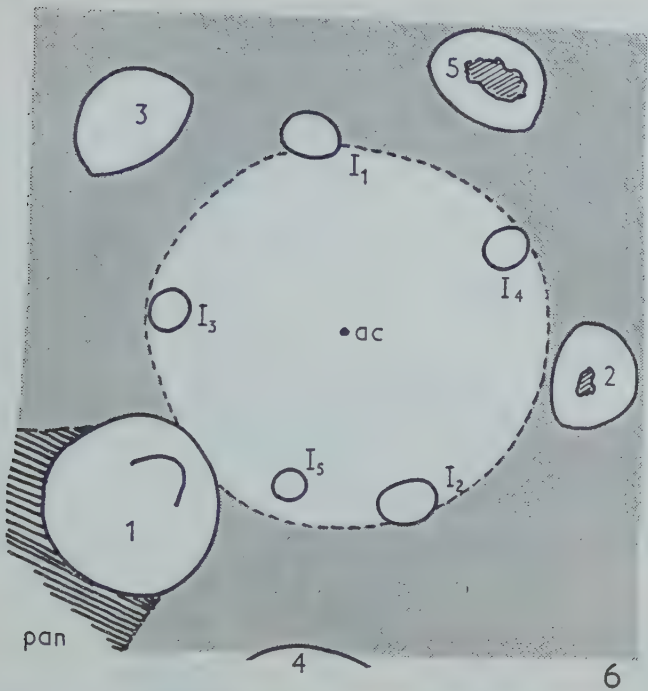


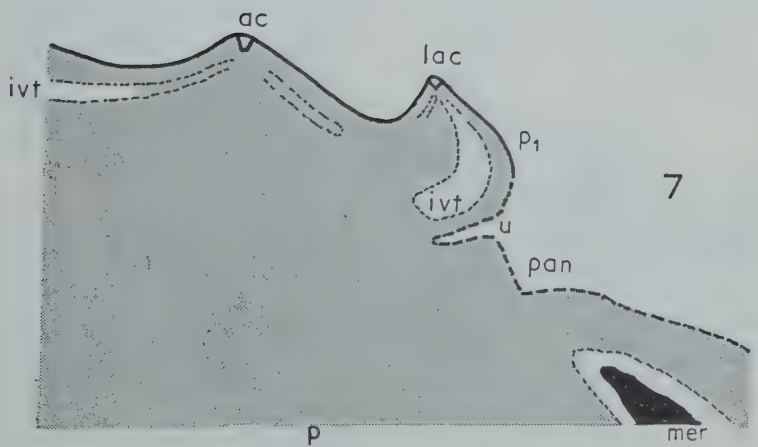
TEXT-FIG. 3. An apex as seen from above in which primordia P_1 and P_2, and primordium site I_1, have been partially isolated as in Experiment (*b*). A leaf primordium with a distended base has been formed at I_1; P_1 and P_2 have grown to abnormally large size with distended basal regions. (×30.)

TEXT-FIGS. 4 and 5. Two longitudinal sections through I_1, in the apex illustrated in Text-fig. 3, showing the conspicuous leaf primordium which has been formed. *ac*, apical cell of shoot; *lac*, leaf apical cell; *u*, undercut; *ivt*, incipient vascular tissue; *sc*, scale. (×55.)

this level there is no such contact, the leaf-trace fading out above the level of the undercut, Text-fig. 11. The serial sections of P_1 yielded similar information, Text-fig. 11. In some apices of this experiment there was evidence of the leaf-trace passing round the inner limit of the undercut. No connexion was made with the shoot vascular tissue below, however, the leaf-trace gradually fading out in the parenchymatous tissue of the cortex, Text-figs. 12–14.

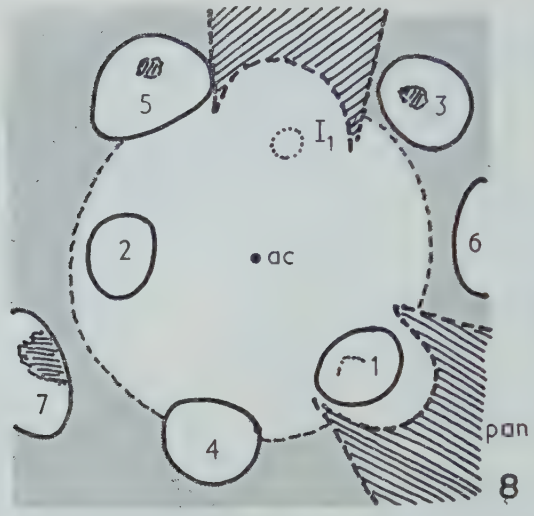
Text-fig. 15 shows an apex in longitudinal section from Experiment (*c*): the I_1 site had been deeply undercut, but radial incisions were not made. An



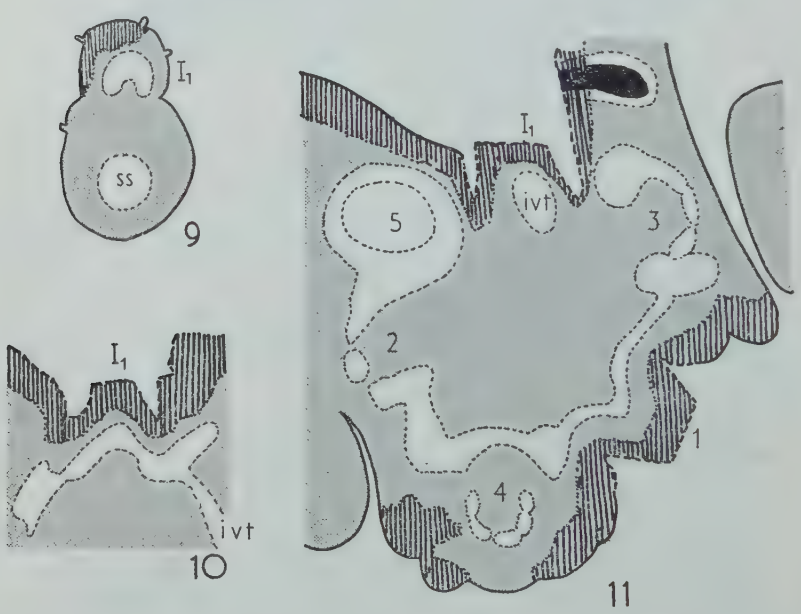


TEXT-FIG. 6. Primordium P_1, isolated as in Experiment (*b*), has developed to large relative size. (×30.)

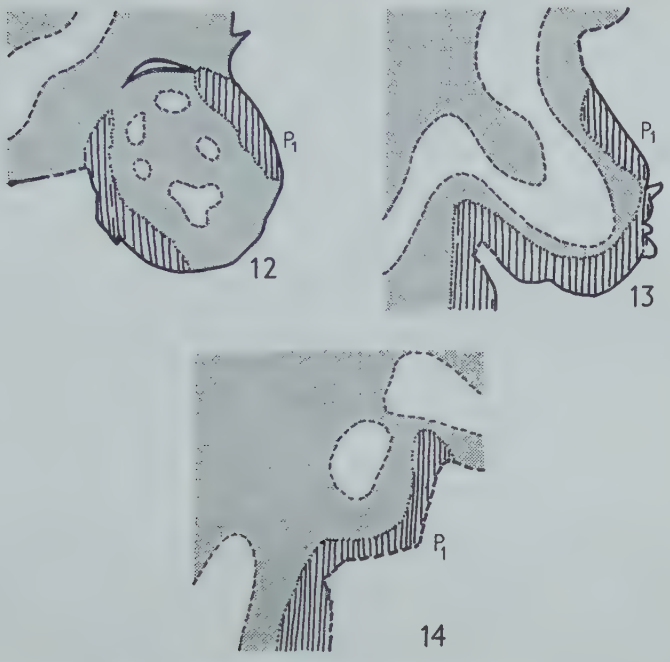
TEXT-FIG. 7. Longitudinal median section of same shoot apex and of P_1; *mer*, meristele of shoot; other indications as in previous illustrations. (×55.)



TEXT-FIG. 8. This also illustrates Experiment (*b*). P_1, and the leaf formed at I_1, have both developed a distended basal region. (×30.)

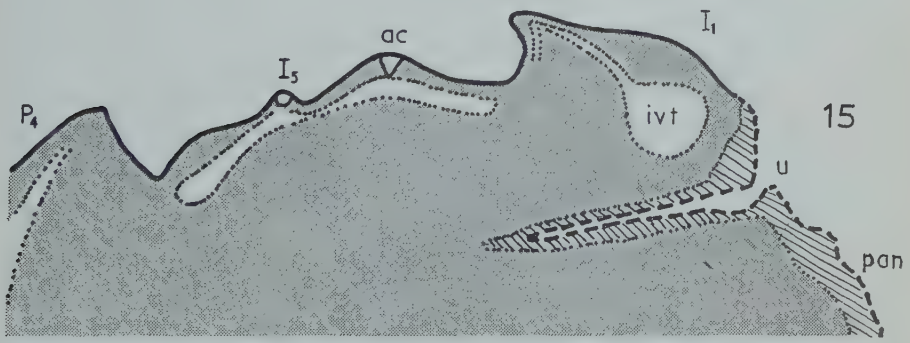


TEXT-FIGS. 9–11. Three serial transverse sections of the apex illustrated in Text-fig. 8. Text-fig. 9, taken near the distal region of the shoot, shows the abnormally large relative size of the I_1 primordium; *ss* incipient shoot stele. Text-fig. 10, taken lower down, shows that the incipient vascular tissue, *ivt*, of I_1 is in lateral continuity with that of the shoot. Text-fig. 11, taken still lower down, shows the vascular tissue of I_1 fading out just above the undercut; the incipient vascular tissue of P_1 (bottom right) is in lateral continuity with that of the axis. Necrotic and darkly staining wound tissue is indicated by the vertical line shading. (×24.)



TEXT-FIGS. 12–14. Three transverse sections in basipetal sequence of a P_1 isolated as in Experiment (*b*).

Text-fig. 12 shows the primordium at the level of insertion of its adaxial side. Text-fig. 13 shows its vascular tissue in median-lateral continuity with that of the axis. Text-fig. 14, taken in the region of the undercutting incision, shows the vascular tissue of the primordium passing round the inner limit of the undercut, before fading out in the cortical tissue lower down. (×37.)



TEXT-FIG. 15. This illustrates the formation of a large primordium in the I_1 position, as in Experiment (*c*). (×15.)

examination of all the sections in the series showed that the vascular tissue of the primordium which developed in this site, though completely separated from the shoot vascular tissue basally, was continuous with it laterally. The illustration also shows that, notwithstanding the interruption of supplies of nutrients by the extensive undercutting, the primordium had grown to large relative size. Pl. III, Fig. 3 illustrates another isolated I_1 in this series of experiments.

Discussion

If the leaf-trace is differentiated in advance of the outgrowing primordium, and indeed determines its inception, it might well be expected that a complete undercutting of a primordium site, with destruction of the underlying incipient vascular tissue of the axis, would prevent the formation of that primordium. The evidence now presented, especially from those experiments in which I_1 and I_2 sites were deeply undercut, is against this view: on the contrary it supports the conclusion from earlier experimental work that a precocious, acropetally differentiating leaf-trace is probably not the factor that determines the inception of a primordium; when the underlying axial pre-vascular tissue is severed, or obliterated, the initial growth of the primordium is not precluded or seriously limited. Esau (1954) had criticized experiments on undercutting primordium sites as a means of testing whether or not acropetally developing leaf-traces determine primordium formation on the grounds that: 'Procambial development in relation to the initiation of leaf primordia in untreated plants did not constitute part of these investigations. Therefore, we have no information whether or not procambium was present beneath the area that was undercut.' The present writer has shown (Wardlaw, 1949*a*) that it is only when a group of the prism-shaped cells of the apical meristem, near its basiscopic margin, begins to grow out forming a low mound—the incipient leaf primordium—that there is evidence of incipient vascular tissue in immediate association with it. The formation of the primordium, and the differentiation of its vascular trace, appear to take place almost simultaneously; both histological developments result from the activity of a growth centre, and, as others have pointed out, may be regarded as two aspects of the same process. In *Dryopteris*, at least, the writer has been unable thus far to detect a precocious leaf-trace associated with I_1 position.

A conspicuous feature of the experiments under consideration is the ability of an undercut primordium, or primordium site, not only to continue its morphogenetic activity, but to undergo a notable enlargement, i.e. when the direct pathway of nutrients from below has been more or less completely interrupted. The partly isolated primordium not only continues to compete successfully with other primordia and with the tissues of the apical meristem for nutrients but may grow to abnormally large size. In this connexion the lateral conjunction of the incipient vascular tissue of the primordium with that of the axis is probably important as affording a means of translocation.

It has already been demonstrated (Wardlaw, 1949*b*) that if a young primordium, or site, is isolated by deep radial incisions from the two adjacent older primordia, it shows rapid relative growth, but, as Wardlaw and Cutter (1954, 1955) have shown, this enhanced rate of growth is not observed if the incisions are shallow and do not sever the incipient vascular tissue. These experimental results may be explained by saying that, in the normal development, the adjacent older primordia are active centres of growth and that they regulate the growth of the younger primordium. This they do by their more active competition for nutrients, and by their production of a growth-regulating substance, the incipient vascular tissue being the means of translocation. An important property of a growth centre lies in its power of drawing nutrients to it. Thus P_1, or I_1, even when isolated laterally and undercut, can still obtain the substances required to sustain primordial growth. These may reach the primordium by one of three paths, namely (i) centrifugally from the developing pith, (ii) laterally, from the pre-stelar tissue of the shoot, above the limit of the radial incisions, or (iii) by way of the adaxial side of the primordium, Text-fig. 2. It may be noted that the undercuts, which were made approximately parallel to the surface of the apical cone, are, after 3–4 weeks of growth, approximately at right-angles to the axis. This is largely a result of the considerable development of medullary tissue. The experimental evidence shows that the primordium, or site, released by the severing and destruction of the vascular tissue from the regulative effects of the adjacent older primordia, which occupy positions lower down on the apex, is still able to obtain the nutrients required to sustain an abnormally rapid relative growth, at least during the early stages of primordial development.

In the several experiments, buds were never obtained unless the apical cell had been damaged; i.e. the expectation that buds or centric leaves might be obtained by one or other of the treatments was not realized. The findings, however, support the thesis that the apical cell (or apical cell group) exercises a regulative effect on the growth and morphogenetic activities of the apical meristem and young primordia. According to this conception, the apical cell group has a distinctive metabolism, the by-products of which move basipetally and regulate the growth of the meristem cells below: cells close to the apical cell grow very slowly and retain their primary embryonic character, but those progressively farther away show an increase in their rate of growth and eventually become differentiated in characteristic ways according to the positions which they occupy; cells at different levels on the apical meristem thus have different competences to react to nutrients and hormonal substances, those lowest down on the apex having the greatest capacity for utilizing carbohydrates and for becoming vacuolated and parenchymatous. Some of the regulated activity and organization which we perceive in the shoot apex must also be attributed to the progressive quantitative and qualitative changes in the nutrient solutions moving upwards from below. Now, in young primordia which have been deeply undercut and isolated laterally,

whereby the direct effects on them of the radial and tangential components of growth in the axis have been greatly reduced, the further development still results in the formation of organs of dorsiventral symmetry, i.e. leaves. These primordia, however, still remain in a normal histological relationship with the upper region of the meristem and with the apical cell group; and although the normal path of nutrients to the primordia has been considerably deflected, the distribution of growth in them is still that characteristic of leaves.

The lateral incisions and the undercuts, by stimulating the formation of wound tissue, may contribute to the abnormally swollen basal region of these primordia.

Acknowledgement

The author wishes to acknowledge the assistance received from Mr. E. Ashby in microscopic preparations and photographic illustrations.

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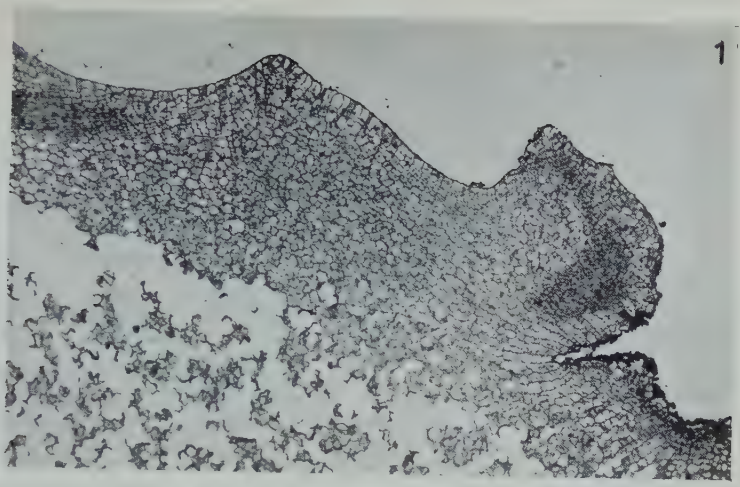
EXPLANATION OF PLATE III

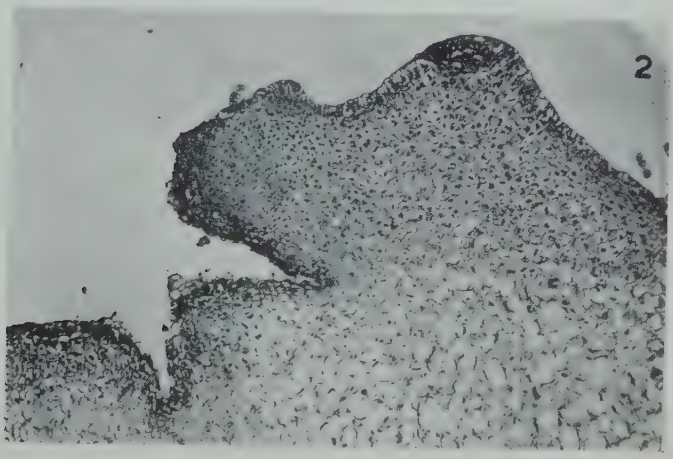
Illustrating Professor C. W. Wardlaw's paper on 'Undercutting Leaf Primordia in *Dryopteris aristata*' (all figures are from untouched photographs).

Fig. 1. Longitudinal median section of a shoot apex and of a partially isolated and undercut P_1 primordium as in Experiment (*b*). The primordium has a conspicuously distended basal region. Its incipient vascular tissue has no continuity with the vascular tissue of the shoot below, but is in lateral continuity with the incipient vascular tissue of the axis. (× 40.)

Fig. 2. Treatment as in Fig. 1 above, Experiment (*b*), showing the formation of a leaf primordium when the I_1 site was undercut and laterally isolated. Note the extensive development of tissue in the basal region of the primordium. (× 40.)

Fig. 3. Longitudinal median section of a shoot apex and a deeply undercut I_1 site, as in Experiment (*c*). A leaf primordium with well-developed pre-stelar tissue and a greatly distended basal region has been formed. The photograph illustrates how the undercutting incision has been displaced to the horizontal position by the growth of files of cells in the pith. (× 100.)





C. W. WARDLAW

Heavy Metal Toxicity and Iron Chlorosis

BY

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ABSTRACT

1. The toxicity of copper, nickel, cobalt, zinc, chromium, and manganese to mustard was studied in water culture, utilizing either the ionic form or the EDTA chelate of the metal in the presence of either ferric chloride or ferric EDTA.

2. In presence of ferric chloride the activity of the metals in producing chlorosis was as given above, i.e. in the order of stability of their chelates. In the presence of ferric versenate, toxicity of the ionic metal was much reduced. The metal chelates gave very little indication of toxicity with either form of iron.

3. It was found that the ratio of total phosphorus to total iron was higher in chlorotic plants than in green plants, irrespective of which metal was causing the toxicity.

4. Copper could be demonstrated in the phloem cells of the root using *bis*-cyclohexanone-oxalydihydrazone as histochemical reagent. It is postulated that transport of iron probably takes place in the phloem as an active process.

5. It would appear that as a major part of the iron in plant cells is attached to nucleo- or phospho-proteins, the heavy metals must be similarly attached to phospho-proteins.

INTRODUCTION

CERTAIN metals are required by plants for healthy growth in very small amounts, and any marked increase causes physiological disturbances, of which a yellowing of the leaves (chlorosis) is usually an apparent symptom. Toxicity attributed to excess of certain metals occurs naturally in various parts of the world; manganiferous soils cause chlorosis in pineapples and other crops (McGeorge, 1923; Fergus, 1954), while plants on soils derived from serpentine rocks may show chlorosis due to excess of nickel in the soil (Hunter and Vergnano, 1952; Minguzzi and Vergnano, 1948; Hunter, 1954). Soils near metal mines or exposed to industrial wastes may be similarly affected (Wallace, Hewitt, and Nicholas, 1945), and the practice of spraying citrus groves over many decades with copper sulphate has caused a toxicity in Florida (Reuther, Smith, and Specht, 1952) and also in vineyards in France (Anne and Dupuis, 1953).

The effect of excess metal on plants appears to be twofold: (*a*) the production of chlorosis, and (*b*) a specific effect of the metal itself (Hewitt, 1948). Hunter and Vergnano (1953) found the order of activity of the metals in producing chlorosis in oats to be $Ni > Cu > Co > Cr > Zn > Mo > Mn$, while Hewitt (1951) showed the activity in sugar-beet, tomato, potato, and kale to be Cd^{++}, Cu^{++}, Co^{++} highly active, and Ni^{++}, CrO_4, Zn^{++}, Cr^{+++}, Mn^{++} less active, in this order. He pointed out that this series presented a reasonable

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close resemblance to the order of stability of the metallo-organic complexes (Mellor and Malley, 1948), which could be stated as follows:

$$Cu^{++} > Ni > Co^{++} > Fe^{+++} > Zn^{++} > Cd^{++} > Fe^{++} > Mn^{++} > Mg^{++}.$$

Arrangement of the elements in a series with regard to specific toxic effects, however, resulted in an order similar to that for induced chlorosis (Hunter and Vergnano, 1953).

The fundamental cause of the chlorosis produced by excess of heavy metals has been attributed to 'induced iron deficiency', since application to the leaf of an iron salt has restored the green colour (Hewitt, 1948). Weinstein and Robbins (1955) have noted that 'a high nutrient level of manganese in the presence of low iron induces a true iron deficiency'. An increase in the amount of phosphorus extractable with Morgan's reagent from the stem with toxic amounts of nickel, cobalt, zinc, chromium, and manganese has been reported (Hunter and Vergnano, 1953; Crooke, Hunter, and Vergnano, 1954), and intensification of metal toxicity symptoms as a result of the application of phosphate has been observed (Crooke and Inkson, 1955; Chapman, Liebig, and Vanselow, 1939; Nicholas and Thomas, 1954), although in the case of cobalt an improvement was obtained if phosphate was applied to the soil (Nicholas and Thomas, 1953).

Of the physiologically active metals, iron undoubtedly plays the major role. Its part in chlorophyll and in haem synthesis has not yet been elucidated, but the two pathways appear to have much in common.

It has recently been shown (DeKock and Strmecki, 1954) that the ratio of total phosphorus to total iron can serve as an index of the iron status of plants; chlorotic plants have high ratios, while plants suffering from iron toxicity have abnormally low ratios; healthy plants have intermediate values. This is found to be the case even at pH values in the alkaline range (DeKock, 1955).

Chelating agents, such as ethylenediamine tetra-acetic acid (EDTA, versene, sequestrene), have been used in horticulture to overcome chlorosis caused by iron deficiency (Stewart and Leonard, 1952; Bould, 1955), and have been shown to reduce toxicity of the excess metal in water culture (Crooke, 1954; Smith and Specht, 1953).

Since high phosphorus-iron ratios have been found even in chlorosis produced by genetical or virological factors, it was desirable to investigate whether these high ratios would be found in chlorosis caused by heavy metal toxicity as indicated in the published work of others (Crooke, Hunter, and Vergnano, 1954; Smith and Specht, 1952). Additional information on the interaction between iron and the heavy metal during absorption and transport could be similarly obtained by using combinations of either the ionic or complexed form of iron or the excess metal.

Heavy metals in excess have a marked effect on the growth of the root, the root system becoming malformed with short curved side roots which may be brown in colour (Chapman, Liebig, and Vanselow, 1939). Smith (1953)

found that copper appears to be held by polar linkages and its distribution shows a direct relation to the iron-replaceable protein nitrogen in the root; thus most of the toxic metal is located at the tips of the roots.

Experimental

About forty mustard (*Sinapis alba*) seeds were sown on plastic gauze supported by wooden rings of about 7 in. in diameter in pots of acid-washed sand and allowed to germinate until the cotyledons were expanded. The rings were then transferred to 5-litre glazed porcelain crocks into which they loosely fitted. Each crock contained 2·50 g. $NaNO_3$; 4·35 g. $Ca(NO_3)_2.4H_2O$; 1·25 g. $MgSO_4.7H_2O$; 2·60 g. K_2SO_4; 1·9 g. $Na_2HPO_4.12H_2O$, with boron, copper, manganese, and zinc added at 0·1 p.p.m. or less. Iron was added at 2 p.p.m. as ferric chloride. The seedlings were thinned to 20 per crock and allowed to grow on for 7 days before differential treatments were applied. Nutrient solutions were supplied with iron at 2 p.p.m. either as the chloride or as the EDTA complex and with nickel, manganese, copper, cobalt, zinc, and chromium added to each form of iron either as the inorganic salt (chloride or sulphate (MX)) or the EDTA complex (MV) at 2 p.p.m. Including 2 ferric chloride and 2 ferric versenate controls there were thus 28 treatments. The solutions were constantly aerated and pH adjusted to 5·5 daily. The plants were harvested after 21 days. Roots were first excised and rinsed in several changes of tap and glass distilled water. Stems and leaves were separated. All samples were dried at 80° C. for 48 hours and ashed in a muffle at 460° overnight.

Colorimetric analyses using a Spekker absorptiometer were made as follows: phosphorus (Williams and Stewart, 1941), iron (Nicholas, 1951), nickel (High, 1945), copper (Riddett, 1953), and manganese (Jones and Leeper, 1951). Zinc, cobalt, and chromium were determined spectrochemically (Mitchell, 1948).

A duplicate experiment, which is not reported here, gave similar results.

Results

The ferric chloride treatments, although showing slight signs of chlorosis, grew well and averaged nearly 4 g. fresh weight per plant. Addition of the ionic salt of the toxic metal brought about a sharp reduction in size and a pronounced chlorosis. The toxicity of the various metals could be listed as follows (average fresh weight being given for comparison):

copper (0·57) > nickel (0·68) > cobalt (0·72) > zinc (1·28) > chromium (3·25) > manganese (3·82).

Each metal, however, did give some evidence of specific toxicity. Thus the copper treatment had dwarfed plants with purple stems and small chlorotic leaves with green veins. The roots were so severely stunted that insufficient material was obtained for analysis. The nickel treatments were characterized

by small plants with chlorotic leaves bearing large white necrotic patches. The cobalt treatment was similar but necrosis was slight. In the zinc treatment, chlorosis was typically golden yellow, whereas with chromium the leaves had green veins. In the manganese treatment chlorosis occurred only as patches along the dentate margins.

Whereas the ferric versenate controls gave strong healthy green plants with an average fresh weight of 6·75 g., addition of the copper salt caused severe chlorosis and stunting (0·54). With nickel added, chlorosis and necrosis were also severe but much less than with the ferric chloride treatments (3·3 g.), while with cobalt the leaves were pale green and there was slight reduction in growth (5·98). With zinc and chromium, the plants were a healthy green in colour and only small reductions in yield were recorded, whereas with manganese there was no sign of any toxicity symptoms, the plants being strong and of a healthy green colour.

Addition of the metal versenate to either the ferric chloride or the ferric versenate treatments gave healthy green plants, but better growth was obtained in all cases when both the metal and the iron were present as the versenates. If free exchange took place between the metal and the iron when either was supplied in the complexed form there would have been no difference between the ferric versenate and ionic metal and the ferric chloride and metal versenate. With copper the difference is very great, showing that exchange between the chelates is slow (Jones and Long, 1952). However, there is apparently some exchange between the copper versenate and the ferric chloride so as to render sufficient iron available to the plant as the chelate without the ionic copper reaching a toxic concentration. This exchange must have been progressively more rapid with nickel, cobalt, zinc, and chromium, while the manganese chelate is completely unstable and exchange would be immediate.

The most marked difference between adding the toxic metal as the ionic salt or the chelate was apparent in the roots. In all cases where the ionic salt was added to the ferric chloride treatments, the roots were stunted, most severely with copper, nickel, and cobalt and less so with zinc, chromium, and manganese in that order. The roots grew normally in the presence of either ferric or metal versenate. However, addition of the metal in ionic form caused accumulation of the metal in the roots, whether in the presence of ferric versenate or ferric chloride, whereas with addition of the metal versenate there was a reduction in the amount of metal in the roots (Table I), dependent on the stability of the chelate (Bersworth Chemical Co.), manganese showing slight reduction and nickel a reduction of some 20 to 50 times. This was reflected in the stems and leaves, little metal appearing in the leaves with application of the metal chelates if the complex was stable; with manganese the difference was slight.

In each treatment in which chlorosis was evident, high phosphorus and/or low iron values were found in the ash of the leaves, giving higher ratios of phosphorus to iron than found in green leaves. With severe chlorosis (copper

TABLE I

Analyses of Roots, Stems, and Leaves of Toxic Mustard Plants

(milligrammes/100 g. ash)

	$FeCl_3$+MX			FeV+MX			$FeCl_3$+MV			FeV+MV			$FeCl_3$ Control			FeV Control		
	R	S	L	R	S	L	R	S	L	R	S	L	R	S	L	R	S	L
Cu	×	×	39·0	×	×	24.0	40·0	38·0	15·0	56·0	39·0	10·0	×	3·2	5·7	×	1·2	0·6
Ni	400	55·6	105·0	237	23·6	52·2	8·0	<0·8	<0·8	11·6	<0·8	<0·8	×	<0·8	<0·8	×	<0·8	<0·8
Co	1540	32·0	125·0	2540	0·8	82·2	240·0	3·4	20·0	230·0	3·2	15·0	0·6	0·1	0·1	0·3	0·05	0·5
Zn	1820	810·0	1470·0	750	150·0	540·0	320·0	60·0	130·0	95·0	55·0	120·0	210·0	65·0	180·0	120·0	30·0	65·0
Cr	2160	1·1	1·5	1590	0·8	1·5	159·0	0·8	5·6	850·0	0·9	6·5	1·9	0·6	0·2	0·9	0·2	0·2
Mn	660	160·0	400·0	250	150·0	440·0	1890·0	62·0	265·0	603·0	50·0	290·0	×	×	×	×	×	×

R = Roots; S = Stems; L = Leaves. MX = Metal chloride or sulphate; MV = Metal versenate. × not obtained.

and nickel) the ratios were high and decreased with decrease in chlorotic symptoms, a good agreement being shown with the order of stability of the metal complexes (Table II).

As the roots are the part of the plant most affected by the heavy metal, and accumulation of the metal is considerable, attempts were made to demonstrate the exact location of the metal in the root using reagents giving distinctive coloured complexes. Most of the reagents tried gave only diffuse colours, as the coloured complexes were soluble or there was sufficient interference from the iron present in the root to make the test unreliable. However, *bis*-cyclohexanone-oxalydihydrazone in dilute ethanol proved to be an excellent

TABLE II

Percentage of Phosphorus and Iron in the Ash of Mustard Leaves affected with Metal Toxicity

Toxic metal	$FeCl_3$+MX			FeV+MX			$FeCl_3$+MV			FeV+MV		
	P	Fe	P/Fe	P	Fe	P/Fe	P	Fe	P/Fe	P	Fe	P/Fe
Cu	3·80	0·058	158	3·00	0·065	46	2·83	0·097	34	3·33	0·100	33
Ni	4·83	0·056	86	3·39	0·071	48	3·14	0·099	32	3·38	0·108	32
Co	3·69	0·062	58	3·49	0·077	45	3·09	0·096	32	3·60	0·103	36
Zn	4·64	0·053	88	3·03	0·106	29	3·99	0·092	37	3·45	0·113	30
Cr	3·42	0·067	51	3·16	0·104	30	3·11	0·105	30	3·16	0·093	34
Mn	3·67	0·081	45	3·57	0·123	29	3·35	0·096	35	3·23	0·100	32
Control	3·01	0·076	40	3·33	0·099	34	3·01	0·076	40	3·33	0·099	34

histochemical reagent for demonstrating the location of copper, a bright blue colour being formed. Results obtained were in agreement with those of Smith (1953), who found most of the copper located in the meristematic zones of citrus seedling roots. The copper is also located in the stele to some extent and crushes of fresh roots showed the phloem units to be deeply stained. Microphotographs clearly show the copper located in the root meristems and stele. The arrested meristems give the toxic root a typical 'barbed wire' appearance, characteristic of metal toxicity.

DISCUSSION

There is little doubt that the major effect of the toxic metal is manifest in the root. Moreover, as the toxic effect of the metals follows closely the order of stability of their chelate complexes, the metal is evidently combined into some protein chelate in the root. This must moreover be the same protein complex into which iron is bound, so that the metal competes for and displaces iron from active chelating centres. This displacement of iron from the root tissues would then depend on the stability of the metal complex relative to iron and its concentration in solution. Thus when the solubility of iron in the nutrient solution was greatly increased by chelation, metal toxicity sharply decreased.

Wallace and North (1953) have shown that the whole ferric versenate complex enters the root. It is considered very likely that further translocation

of the iron takes place in the phloem, which is where copper is mainly concentrated in the root and where it would thus exert maximum interference. Using a split-root technique it was shown that transport of iron and other metals must be an active process, as roots in toxic solutions caused little translocation of iron and metal to the leaves. As divalent metals such as copper and nickel which are strongly chelated, are not absorbed or scarcely so, absorption of the iron versenate may depend upon its trivalent state. In support of this view, it has been found that more aluminium is translocated to the leaves of plants when supplied as the versenate than as the chloride (unpublished experiments). However, our knowledge of the transport of iron and other metals to the leaves is far from complete.

The major part of the iron in the leaf is located in the chloroplasts and is there attached by phosphate groups to phosphoproteins (Noack and Liebich, 1940), a small amount of the free iron being in the ferrous state and physiologically active. Reduction in iron supply to the leaf brings about a rapid fall in the free iron and a consequent increase in the phosphorus-iron ratio.

It would appear that the heavy metals present in the cell are similarly attached to phosphoproteins. Bolas and Ruck (1955) found that the manganese and magnesium contained in rooted leaves were not transported into the developed shoot. Ramamoorthy and Desai (1946) noted that injection of ferrous sulphate into one-half of a chlorotic tobacco leaf caused the toxic level of manganese to decrease sharply as the leaf greened. Similarly when chlorotic pear leaves were greened by application of ferrous sulphate to the roots, the phosphorus content of the mature leaves was decreased (Lindner and Harley, 1944). Iron therefore appears to control the amount of phosphoprotein and the attached metals. This is probably the basis of the metal-iron ratio (Crooke, Hunter, and Vergnano, 1954) in metal toxicity.

Source of nitrogen either as nitrate or urea may markedly affect the toxicity induced by a metal (Hewitt, 1950). This would be connected with protein synthesis as a carrier of iron, as increase of nitrogen leads to a lowering of the phosphorus-iron ratio (Smith, Reuther, Specht, and Huncair, 1954), while increase of phosphorus leads to a decrease in total nitrogen (Chapman, Liebig, and Vanselow, 1939). Nickel in toxic concentrations has been found to cause a reduction in the nitrogen status of oats (Crooke and Inkson, 1955).

From the above studies and those reported elsewhere chlorosis would appear to follow a general biochemical pattern irrespective of whether it is 'lime-induced' (DeKock, 1955) or caused by iron deficiency (DeKock and Strmecki, 1954), genetical or virological factors (DeKock and Hall, 1955), or heavy metals.

Acknowledgements

Mr. A. Hall, Miss D. Montgomery, and Miss A. Adams were responsible for the phosphorus, iron, copper, nickel, and manganese analyses presented here. The author is indebted to the Department of Spectrochemistry for the zinc, cobalt, and chromium analyses.

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Experimental and Analytical Studies of Pteridophytes

XXXIII. The Experimental Induction of Buds from Leaf Primordia in *Dryopteris aristata* Druce

BY

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With Plates IV and V and thirty-two Figures in the Text

Abstract

When a young leaf primordium of *Dryopteris aristata* is isolated on a plug of tissue at any time prior to the formation of a lenticular apical cell, the tissue may be induced to develop as a solenostelic bud instead of a leaf. The result depends on the rate of leaf development. In some apices, leaf determination may take place during the first plastochrone; in others it may be postponed until the end of the third plastochrone. Isolation of very young primordia by deep incisions in close proximity to the primordium may result in the cessation of meristematic growth and the formation of parenchyma. The vascular structure of the isolated primordia and the phyllotaxis of the induced buds are described, and the significance of the findings is discussed.

Introduction

THE influence of the shoot apex upon the formation, growth, and development of the leaf primordia to which it gives rise may be experimentally investigated either by destroying the apex or by isolating leaf primordia or presumptive leaf positions from it. In *Dryopteris aristata* Druce (*D. austriaca* (Jacq.) Woynar), Wardlaw (1949*a*, 1949*b*) made the important discovery that leaf and bud primordia are histologically identical and that buds may be caused to develop at presumptive leaf positions when these are isolated from the shoot apex by a deep and wide tangential incision. Preliminary exploratory experiments on the isolation of young emergent leaf primordia resulted in the development of awl-shaped leaves of limited growth and approximately radial symmetry, with associated axillary buds (Wardlaw, 1947, 1949*c*). In these experiments primordia were isolated by close incisions, and the primordia isolated were often relatively advanced, e.g. P_4–P_9 (Wardlaw, 1949*c*). In the course of further experiments reported below, which were originally undertaken to investigate the effect of the size of the isolated plug of tissue upon the growth of the subtended leaf (Wardlaw, 1949*c*), it was found that under certain conditions it was possible to cause the three youngest visible leaf primordia to develop as buds. By means of these experiments it has been possible to correlate the irrevocable determination of a primordium as a foliar organ with a visible histological phenomenon, namely the formation of an enlarged, lenticular apical cell.

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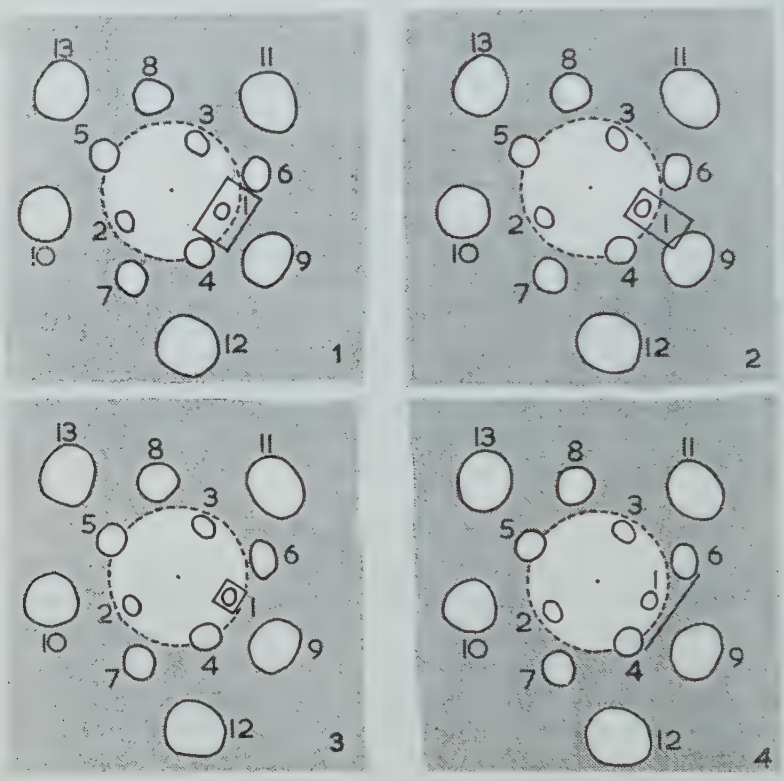
Part of this work has already been briefly reported (Cutter, 1954); it is now illustrated and more fully described and discussed.

Materials and Methods

Large shoot apices of mature plants of *Dryopteris aristata* were laid bare as described previously (e.g. Wardlaw, 1944), the pieces of rhizome being main-



Text-figs. 1–4. Diagrams showing the position of the incisions.
Text-fig. 1. Four incisions, plug large, elongated in a direction tangential to the shoot apex.
Text-fig. 2. Four incisions, plug large, elongated in a direction radial to the shoot apex.
Text-fig. 3. Four incisions, plug small and symmetrical.
Text-fig. 4. Single abaxial incision.

tained in pans of damp peat. The pans were kept in the laboratory during the growing season, but were sometimes transferred to a room maintained at a constant temperature of 21°±1° C. during the winter months to accelerate growth.

Leaf primordia were isolated from the shoot apex and adjacent primordia by four deep vertical incisions, which penetrated some distance into the pith. The majority of the isolated plugs of tissue were relatively large, and rectangular in surface view, elongated in a direction either tangential (Text-fig. 1) or radial (Text-fig. 2) to the shoot apex. Additional experiments were carried

out in which the cuts were made close to the leaf primordium, the isolated plugs being of the type shown in Text-fig. 3.

The terminology used in describing leaf primordia and presumptive leaf sites is that of Snow and Snow (1931). The term *determination* is here used to indicate the fixation of the fate of a group of cells (Needham, 1942).

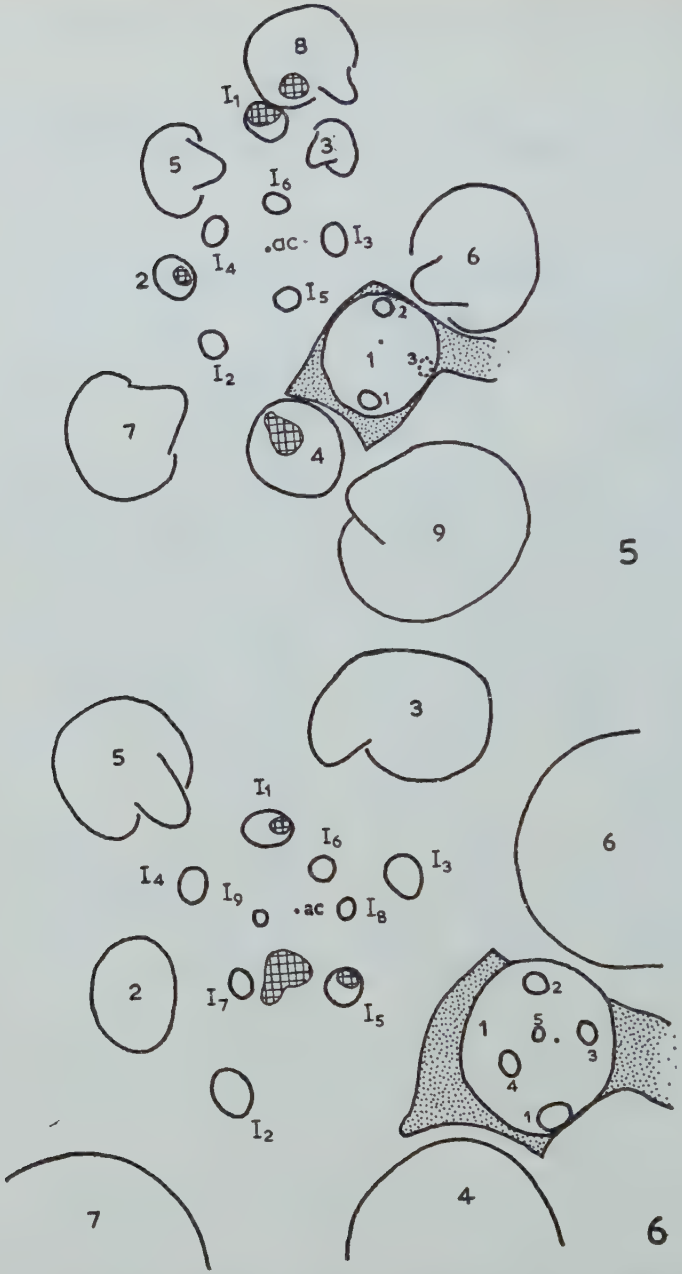
Experimental Observations

(i) *Isolation on large plugs of tissue*

Experiments were carried out on primordia P_1–P_4 during the ten months from March to December, 1953, and resulted always in the development either of a bud (Pl. V, Figs. 1–3 and Text-figs. 5–8) or of a normal dorsiventral circinate leaf primordium (Text-fig. 9). An analysis of the experimental results is presented in Table I. Isolation of the primordium from the shoot apex by a single wide and deep adaxial incision was sufficient to cause its development as a bud, as Wardlaw (1949*a*, 1949*b*) had already demonstrated in the case of the I_1 position. Similar deep abaxial incisions (Text-fig. 4) had no effect on the development of the primordium.

These results show that in large apices the three youngest leaf primordia may all be capable of development as shoot buds bearing leaf primordia (Pl. V, Figs. 1–3 and Text-figs. 5–8) if they are isolated from the shoot apex by a deep incision of considerable width. While Table I gives a reliable indication of the ratio of buds to leaves which may be expected from isolations of P_1 under the specified conditions, in many cases the examples of P_2 and P_3 were chosen because their external appearance indicated that they might be capable of development as buds. The results for P_2 and P_3, therefore, do not give a reliable estimate of the proportion of buds to be expected from random isolations of these primordia.

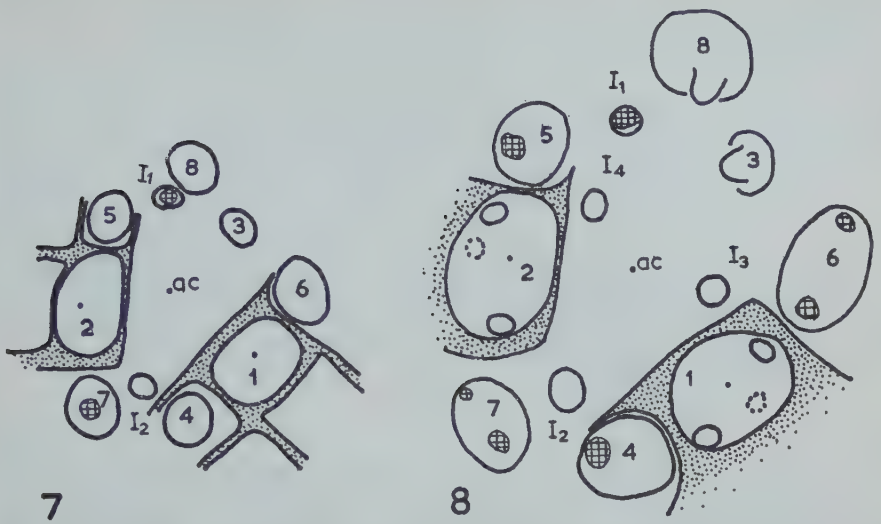
Since it is known that pronounced seasonal metabolic changes occur in the apex of *D. aristata* (Frazer, 1946), it was thought that the season of collection of the specimens might have an effect upon the development of the isolated primordia. An analysis of the monthly results for isolations of P_1, however, showed that these changes had no significant effect within the limits of the small numbers involved. The percentage of buds obtained from the monthly collections varied from 80 to 100 per cent. Under the conditions in which the specimens were maintained, the average length of plastochrone for the collections from which 100 per cent. bud formation was obtained, namely June, October, and December, was 6·9, 11·4, and 11·0 days respectively, indicating very different rates of growth. It appears, therefore, that neither the rate of growth of the apex nor the seasonal changes in its metabolism affects the morphological development of P_1 primordia isolated by deep incisions. Any such effects, however, might well be more readily observable on P_2 and P_3 primordia; the numbers of such primordia isolated in the course



TEXT-FIGS. 5 and 6. An apex in which P_1 was isolated on a large plug of tissue by four deep incisions.

Text-fig. 5. Forty days after isolation of P_1. A bud with three leaf primordia has developed on the P_1 plug (leaf primordia numbered in the order of their formation). I_1–I_6 present. *ac* = apical cell. (Compare Pl. V, Fig. 1.)

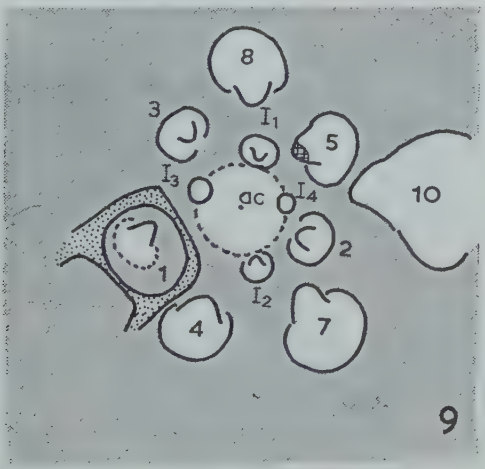
Text-fig. 6. Eighteen days later. The bud now has five leaf primordia. The dotted area shows the gaping of the incisions; necrosed tissue cross-hatched. (× 14.)



TEXT-FIGS. 7 and 8. A specimen in which P_1 and P_2 were isolated by four deep incisions on large plugs of tissue. (Compare Pl. V, Figs. 2 and 3.)

Text-fig. 7. Seventeen days after isolation of P_1 and P_2. Large dome-shaped swellings have developed on the plugs.

Text-fig. 8. A fortnight later. Buds with three leaf primordia have developed from P_1 and P_2. Four new leaf primordia (I_1–I_4) are present on the shoot apex. The dotted area indicates the gaping of the incisions; necrosed tissue cross-hatched. *ac* = apical cell. (×14.)



TEXT-FIG. 9. A specimen in which P_1, after isolation on a large plug of tissue by four deep incisions, developed as a normal dorsiventral, circinate leaf primordium. The tip of P_1 is in approximately normal size relationship with the other primordia, but there is considerable basal growth over the whole plug of tissue. Subsequently P_1 was outgrown by younger primordia. Thirty-seven days after isolation of P_1. The sub-apical region is stippled, and the dotted area indicates the gaping of the incisions. *ac* = apical cell. (Tracing from a photograph.) (×14.)

of this work were rather small for comparison on a seasonal basis, but the results obtained do not suggest that there was a seasonal effect.

TABLE I

Experimental Isolations of Leaf Primordia by Deep Vertical Incisions on Large Plugs of Tissue

Primordium isolated	Total no. of viable isolations	No. which developed as buds	No. which developed as leaves	No. which developed as leaves, or moribund leaves, with associated axillary buds
P_1	120	111	9	—
P_2	27	20	6	1
P_3	10	2	7	1
P_4	7	—	5	2

From Table II it is evident that leaf primordia isolated at a later stage of the plastochrone yield a higher proportion of leaves than those isolated at an earlier stage. Careful observation of leaf ontogeny has shown that the distinction can be rendered more precise. The first stage in leaf formation is marked by the outgrowth of a group of equivalent prism-shaped cells of the apical meristem (Wardlaw, 1949*c*); subsequently one of these cells, near the centre of the locus of growth, enlarges and grows out, taking precedence over the other cells. Ultimately this cell becomes very much enlarged, and its

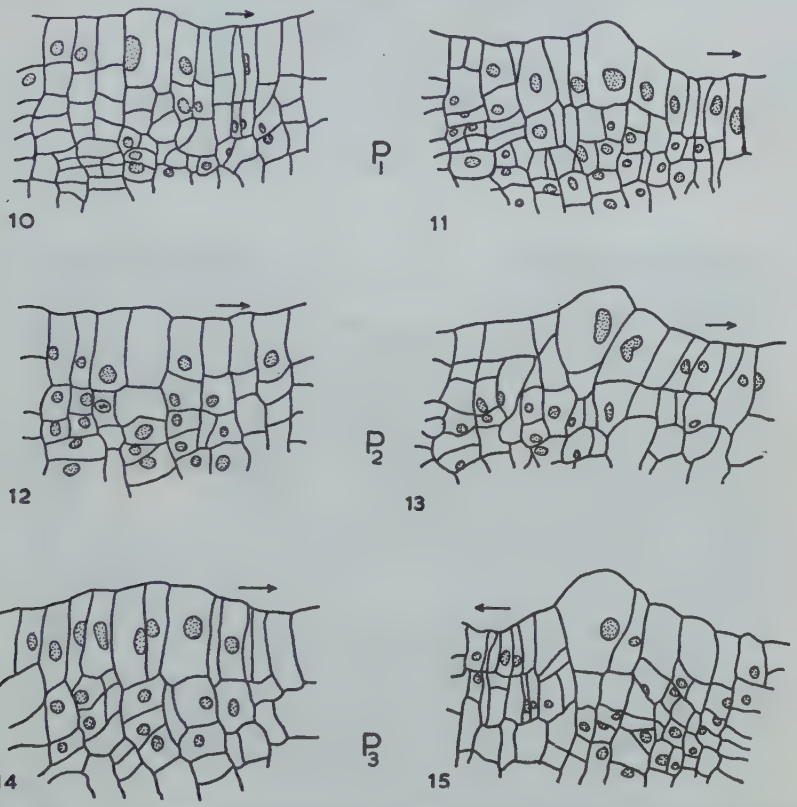
TABLE II

The Effect of the Stage of the Plastochrone Attained at the Time of Isolation on the Subsequent Development of P_1 Primordia Isolated on Large Plugs of Tissue by Deep Vertical Incisions

Stage of plastochrone	Total no. of viable isolations	No. which developed as buds	No. which developed as leaves	% Buds	% Leaves
Early . .	37	36	1	97·3	2·7
Middle . .	58	56	2	96·6	3·4
Late . . .	25	19	6	76·0	24·0

lateral walls become slightly convex; at this stage it is recognizable as the characteristic lenticular apical cell of the leaf of this species. The cellular configuration of the leaf primordia can be distinguished in most apices under a binocular microscope. From such observations it was found that before a single cell has visibly taken precedence over the other cells of the primordium and has grown out from the surrounding tissue, a primordium is capable of development as a bud when isolated from the shoot apex by a deep incision, but after a single cell has become dominant and is beginning to become lenticular in shape, the primordium is no longer able to develop as a bud, and, under the conditions of these experiments, will develop as a normal dorsiventral leaf primordium. Determination of the primordium as a leaf therefore

coincides with the formation of an enlarged, lenticular apical cell. The stage at which a primordium attains this condition is variable in different apices; an enlarged cell which is becoming lenticular may be present at the end of the first plastochrone, or its formation may be delayed until the end of the third or possibly even the beginning of the fourth. This has been confirmed by anatomical investigation (Text-figs. 10–15). Thus it is that even a primordium in its third plastochrone may still be capable of development as a bud.

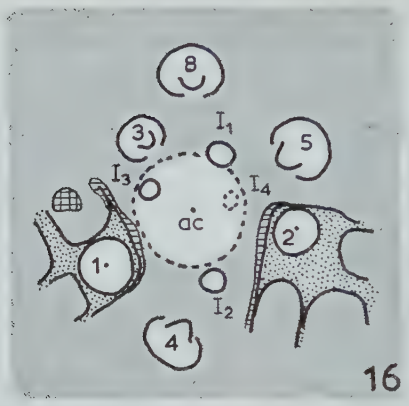


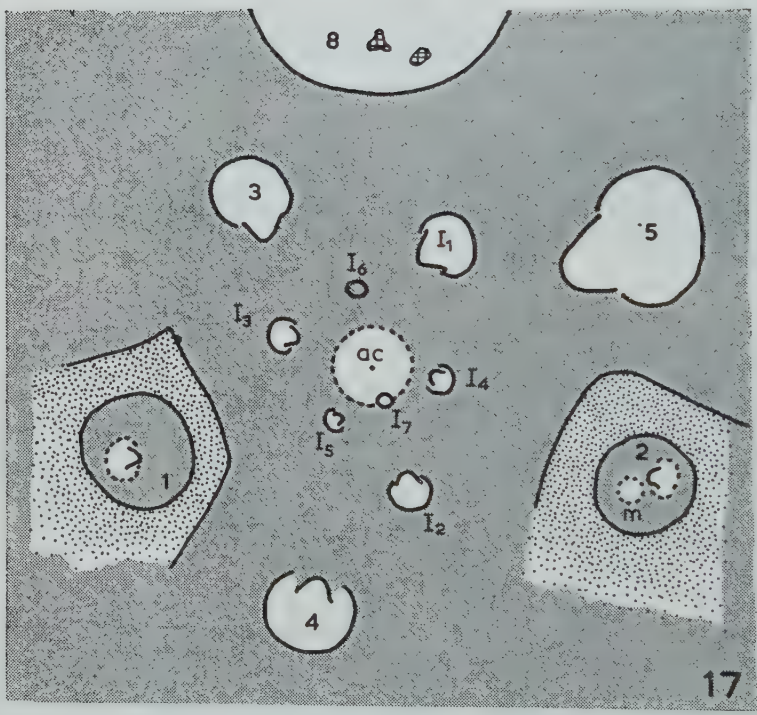
TEXT-FIGS. 10–15. Longitudinal sections of young leaf primordia in the plane of the shoot apex.

Text-figs. 10, 12, and 14—primordia which would be capable of development as buds if isolated from the shoot apex by a wide and deep tangential incision; Text-figs. 11, 13, and 15—primordia which would no longer be capable of development as buds. An enlarged apical cell may be present at the P_1 stage, or it may still be absent at the P_3 stage. The arrows indicate the direction of the shoot apex. Nuclei stippled. (×142.)

(ii) *Isolation on small plugs of tissue*

Experiments were also carried out in which four deep vertical incisions were made close to the primordium (Text-fig. 3). The cuts were probably still not as close to the primordium as those of Wardlaw (1947, 1949*c*); nevertheless the results are in broad agreement with his. The isolated primordia underwent less growth than those on larger plugs, and many of the younger





TEXT-FIGS. 16 and 17. An apex in which P_1 and P_2 were isolated by four deep incisions on small plugs of tissue.

Text-fig. 16. Forty-three days after isolation. P_1 and P_2 have developed as conical organs which are as yet of radial symmetry.

Text-fig. 17. One hundred and six days after isolation. P_1 and P_2 have now become dorsi-ventral and circinate. P_2 has a small axillary meristem (*m*). The isolated plugs have grown considerably in height, but the tips of P_1 and P_2 are relatively small compared with I_1, I_2, &c. The sub-apical region is stippled, and the dotted area indicates the very marked gaping of the incisions. Necrosed tissue cross-hatched; *ac* = apical cell. (Tracings from photographs.) (× 14.)

primordia became moribund (Wardlaw, 1949*c*), the isolated plug of tissue developing as a parenchymatous mound (Table III). This result is quite distinct from that obtained by isolating comparable primordia with shallow cuts, which also resulted in cessation of meristematic growth (Wardlaw and

TABLE III

Experimental Isolations of Leaf Primordia and Presumptive Leaf Positions on Small Plugs of Tissue by Four Deep Vertical Incisions

Primordium isolated	Total no. of viable isolations	No. which developed as buds	No. which developed as leaves	No. which developed as parenchymatous mounds
I_1	8*	2	—	5
P_1	22	7	3	12
P_2	11	5	5†	1
P_3	1	—	1	—

* One isolated I_1 primordium developed as an awl-shaped, conical organ of radial symmetry. † One specimen later developed a small axillary meristem.

Cutter, 1954, 1955); for in the present instance the isolated tissue actually grew out and formed a parenchymatous swelling, whereas with shallow incisions the primordia disappeared and there was no vertical growth of the subtending plug of tissue.

Primordia which developed as leaves when isolated on small plugs grew at

TABLE IV

The Effect of the Stage of the Plastochrone Attained at the Time of Isolation on the Subsequent Development of P_1 Primordia Isolated on Small Plugs of Tissue by Four Deep Vertical Incisions

Stage of plastochrone	Total no. of viable isolations	No. which developed as buds	No. which developed as leaves	No. which developed as parenchymatous mounds
Early . .	15	3	1	11
Middle . .	6	4	1	1
Late . .	1	—	1	—

first as conical organs of approximately radial symmetry (Text-fig. 16). After a considerable time had elapsed, the tips became dorsiventral and circinately curved, but the tips were rather small and not markedly circinate (Text-fig. 17).

It is noteworthy that the majority of primordia which became moribund and underwent parenchymatization consisted of I_1 and early plastochrone P_1 primordia (Tables III and IV).

(iii) *Isolation by other systems of incisions*

It is possible to provide an isolated primordium with a plug of large surface area, but with the restricted nutrition of a small plug, by isolating it

with deep converging undercuts. Preliminary experiments of this sort showed that primordia so isolated were still capable of development as buds, although their development was greatly retarded and only one leaf primordium was present after periods of up to 14 weeks. Indeed, in some cases development did not proceed beyond a domed swelling of a rather unhealthy appearance during the period of observation. Similar experiments in which the primordium was isolated on an asymmetrical plug undercut on the longer axis gave comparable results. None of the isolated primordia developed as a parenchymatous mound or as a leaf primordium. Primordia isolated by deep diverging incisions, so that the base of the plug was of greater area than the top, developed either as buds or as parenchymatous swellings undergoing little growth. It appears, therefore, that it is the proximity of the cuts to the primordium in the superficial regions of tissue rather than a restriction of its nutrient supply which leads to its parenchymatous development. These experiments, however, were of a preliminary nature and were few in number; confirmatory evidence is required.

(iv) *Growth of the isolated plugs*

Growth took place over the whole undamaged area of those plugs which would give rise to buds, the vertical or axial component of the new organ remaining greatest at the centre of the original primordium irrespective of its position on the plug. The organ first became slightly domed, then more conspicuously so, and finally conical; the inception of leaf primordia on the bud usually occurred when it was markedly domed (Pl. V, Figs. 1–3 and Text-figs. 5–8). In those plugs in which the primordia developed as leaves, however, growth remained localized at the position of the original primordium, much less growth occurring over the rest of the plug, which eventually gave rise to scales; that is to say, on plugs which bore leaves meristematic growth was largely confined to the original primordium, whereas on those which gave rise to buds it extended over almost the whole area of the plug. Growth in height of all the plugs, both large and small, was considerable and rapid; due to this, isolated leaf primordia attained a greater height than older primordia, but the tip of the leaf primordium was nevertheless of about normal size, although isolated leaves might be more extensive basally (Text-fig. 9). Growth of both the radial and tangential axes of the isolated plugs was usually considerable and approximately equal in extent, resulting in the cylindrical form of the plugs; such growth was sometimes slightly more and sometimes less rapid than that of the subtending shoot apex, as measured by radial extension. When the growth rate of the isolated plugs was less than that of the surrounding tissue, the incisions gaped open. Growth of the small plugs bearing young leaf primordia which became parenchymatous was slight and was characterized by the outgrowth of numerous scales.

Although isolated leaf primordia remained in normal size relationship with older and younger primordia for a time (Text-fig. 9), ultimately growth slowed down very considerably and younger primordia surpassed them in size. One

isolated leaf primordium was allowed to grow on for 5 months; at the end of 4 months it had become pinnate, but was smaller than I_6. Moreover, the buds which developed from primordia isolated by four deep incisions, although potentially capable of unlimited growth, in fact never grew out and subtended expanded leaves; this is in contrast to the much smaller buds which frequently arise on the petiole bases of small expanded leaves when the specimen has been kept in culture for some time. In the case of one specimen, kept under observation for a lengthy period (that illustrated in Pl. V, Fig. 1 and Text-figs. 5, 6, and 18), the bud which developed from an isolated P_1 bore six leaf primordia 8 weeks after isolation; 42 weeks after isolation 10 leaf primordia were present, and no new leaf primordia had been formed for at least 15 weeks.

(v) *Marking of the isolated primordia*

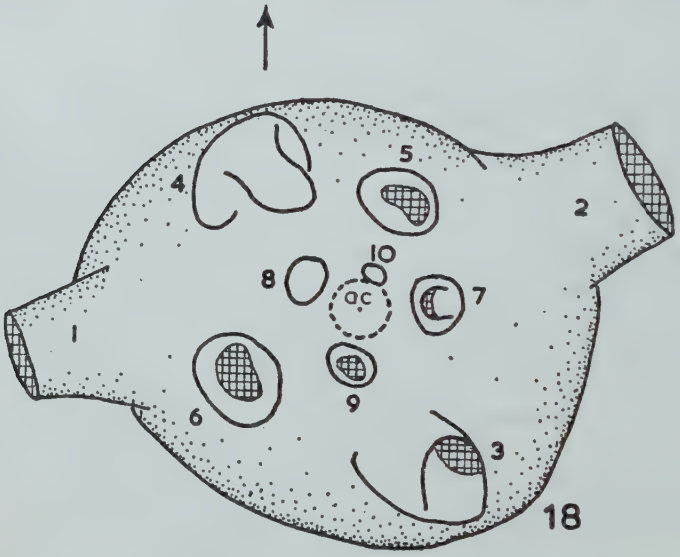
In order to determine with certainty whether the apex of the isolated leaf primordium did in fact give rise to the apex of the induced bud, attempts were made to mark the centre or, where visible, the apical cell of the primordium at the time of isolation with a free-flowing brand of indian ink (Pelikan), applied with a small paint brush from which the majority of the bristles had been removed. By this method it was possible to achieve marks small enough to label single cells, but due to the technical difficulties involved it was not always found possible accurately to mark the central cell of the leaf primordium, and any initial discrepancy between the mark and the centre of the primordium became magnified as a result of growth and cell division. Due to cell division, also, the marks frequently became considerably broken up and dispersed. However, a few of the marks which were originally quite central remained visible long enough to be observable directly on the apex of the induced bud after the inception of its first leaf primordia, and it was invariably found that the ink mark occupied the same position relative to the apex of the induced bud as it had done relative to the apex of the isolated leaf primordium. It therefore seems certain that the apex of the isolated leaf primordium does give rise to the apex of the bud. No toxic effects of the ink were observed.

(vi) *Phyllotaxis of the induced buds*

The inception of leaf primordia on the induced buds usually took place when the bud was conspicuously domed, generally about 3 to 4 weeks after isolation. The first leaf primordium always arose on the longer axis of the isolated plug; thus on plugs of the type shown in Text-fig. 2 the first leaf primordium arose in an abaxial position, and on plugs of the type shown in Text-fig. 1—those used in the majority of the experiments—leaf primordia arose first on the axis of the plug tangential to the shoot apex. In P_1 plugs of this type, where the primordium was symmetrically situated on the plug, two leaf primordia frequently arose almost contemporaneously in approximately opposite positions towards P_4 and P_6. Although the specimens were

observed at weekly intervals, it was frequently impossible to be certain which was the older of these two primordia. Out of forty-nine P_1 buds in which it was possible to observe with complete certainty which was the older of the first two leaf primordia, in thirty-seven (75 per cent.) the first leaf primordium arose on the P_4 side of the plug, that is, towards the younger flanking primordium. Similar results were obtained from observations of P_2 buds.

The first two leaf primordia of an induced bud were thus always approximately opposite; the next two were again almost opposite, not quite at right angles to the preceding pair (Text-fig. 18). These primordia generally did not arise simultaneously. Normal spiral phyllotaxis usually was not established

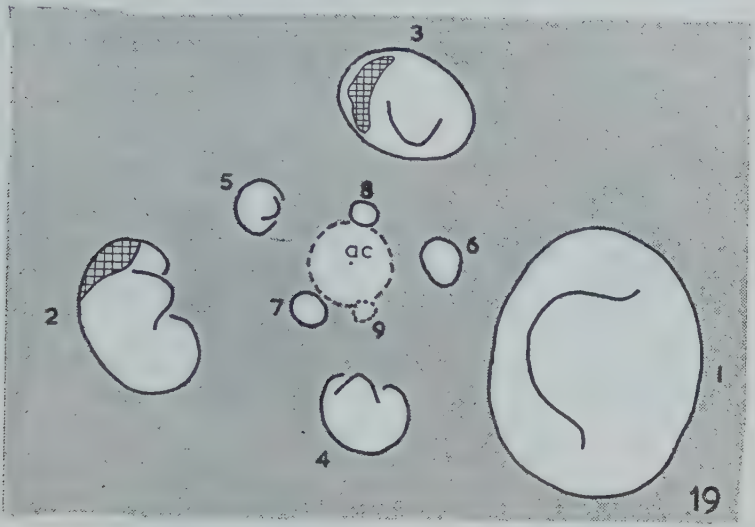


TEXT-FIG. 18. Surface view of a bud which developed from an isolated P_1 (that illustrated in Pl. V, Fig. 1 and Text-figs. 5 and 6), showing the phyllotactic system. Normal spiral phyllotaxis was not established until after the formation of six leaf primordia (primordia are numbered in the order of their formation). Forty-two weeks after isolation of P_1. The arrow indicates the direction of the shoot apex. *ac* = apical cell; necrosed tissue cross-hatched. (×27.)

until after the inception of at least six leaf primordia (Text-fig. 18). Weisse (1897), van Iterson (1907), and Snow and Snow (1931) report the approximately opposite arrangement of the first two true leaves in the seedlings of various angiosperms; Snow and Snow also state that the divergence angle of the first six primordia in *Lupinus albus* is variable, oscillating above and below the normal value. Van Iterson (1907) describes angiosperm seedlings in which several decussate pairs of leaves may occur before the transition to spiral phyllotaxis takes place; the condition in *Ulex europaeus* bears a rather striking resemblance to the phyllotaxis of these induced buds of *Dryopteris aristata* (see van Iterson, 1907, Fig. 80, p. 279).

It is noteworthy that, in contrast to the condition in buds induced in

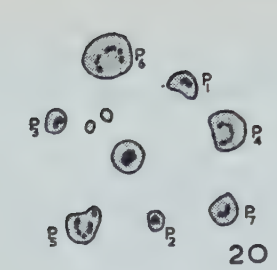
D. aristata by deep cuts, buds which arise on a damaged shoot apex, or those induced by isolating I_1 or P_1 positions by shallow cuts and damaging the shoot apex, may possess normal, or approximately normal, spiral phyllotaxis from their inception (Text-fig. 19).

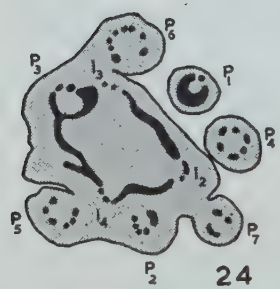


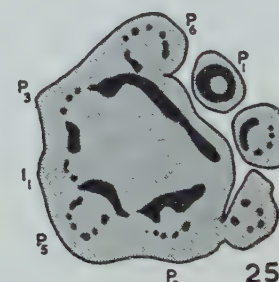
Text-fig. 19. Surface view of a bud which arose at P_1 when P_1 was isolated by four *shallow* incisions and the shoot apex damaged, showing the approximately normal spiral phyllotaxis. Leaf primordia are numbered in the order of their formation. Compare Text-fig. 18. *ac* = apical cell; necrosed tissue cross-hatched; sub-apical region stippled. (×27.)

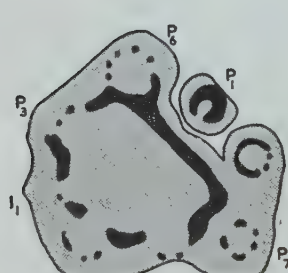
Anatomical Observations

The vascular systems of the induced buds were always solenostelic (Pl. VI, Figs. 4 and 5); a dictyostele was formed in those buds which were allowed to grow on and form several leaves. The vascular structure of the leaves which developed on large plugs was as follows. At the tip of the leaf primordia, a crescentic band of prestelar or incipient vascular tissue was present (Text-figs. 20 and 21); on proceeding basipetally, discrete strands of prestelar tissue were present abaxially, as in the case of untreated leaf primordia, but a continuous strand of prestelar tissue was present adaxially (Text-figs. 22–24). The latter was also found in older primordia, e.g. P_4 or P_6, which had been partially isolated from the apex by extensions of the cuts (Text-figs. 25 and 26, and Pl. VI, Fig. 4). It may be that removal of tension has here led to the development of prestelar tissue from tissue which would normally have developed as parenchyma as a result of factors of stress (Wardlaw, 1945*a*, 1947). At a still lower level the vascular system was usually solenostelic (Text-fig. 25), but this region was occasionally lacking. Presumably such solenosteles must have resulted from the medullation, due to the stresses set up during growth, of the prestelar tissue which extended over the whole area of the plug at the time of isolation (see Wardlaw, 1947, Fig. 15).









TEXT-FIGS. 20–27. Basipetal serial transverse sections of an apex in which P_1, isolated by four deep incisions on a large plug of tissue, developed as a normal dorsiventral leaf primordium.

Text-figs. 20 and 21. Normal leaf vascular structure in P_1.

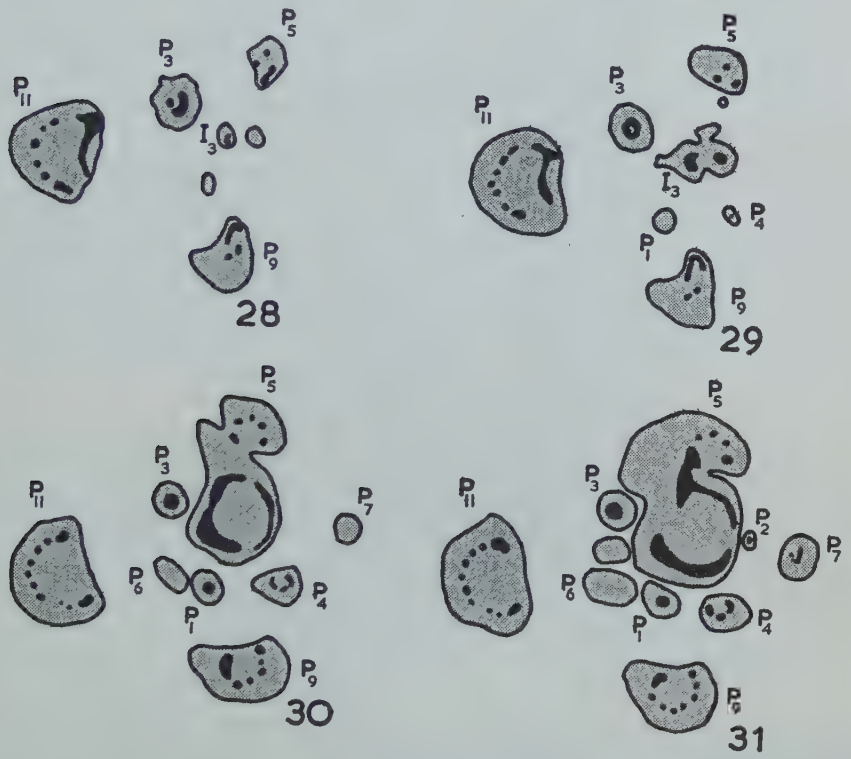
Text-figs. 22–24. A continuous strand of prestelar tissue (i.e. incipient vascular tissue) is present adaxially in P_1.

Text-fig. 25. P_1 is solenostelic.

Text-figs. 26 and 27. The adaxial face of the solenostele in P_1 fades out.

Note the continuous adaxial strand of prestelar tissue in P_4, which has been partially isolated by the cuts, in Text-figs. 25 and 26. Prestelar tissue black. (×7.)

In all large isolated plugs, whether they gave rise to leaves or buds, on proceeding basipetally the adaxial face of the solenostele faded out, leaving a crescentic area of prestelar tissue in an abaxial position, which finally split into two discrete strands and ended blindly in the cortex (cf. Wardlaw, 1947, Fig. 16). No contact in a vertical direction was ever effected with the shoot stele. Out of twenty buds examined in transverse section, two made some



TEXT-FIGS. 28–31. Basipetal serial sections of an apex in which P_1 and P_3 were isolated by four deep cuts on small plugs of tissue. P_1 developed as a parenchymatous outgrowth, P_3 as a dorsiventral leaf. Both are protostelic at the base; the vascular tissue of P_1 fades out higher up; the vascular structure of P_3 becomes that of a normal leaf primordium abaxially, with a continuous strand of prestelar tissue adaxially. Prestelar tissue black. (× 10)

lateral contact with other prestelar tissue. In one case the basal part of the bud stele became connected laterally with the stele of a bud abaxial to it; in the other the bud stele conjoined laterally with axial prestelar tissue, but this connexion was lost at the leaf gap of P_9 and ultimately the bud stele ended blindly in the cortex. Wardlaw (1949*c*, 1950) has shown that shoot apices of *D. aristata* isolated by deep vertical incisions also never establish vascular connexion with axial stelar tissue.

The vascular system of primordia which developed as leaves or as parenchymatous outgrowths when isolated on small plugs was basally protostelic. In

those which developed as leaves a small discrete strand of prestelar tissue in an abaxial position and a continuous crescentic strand in an adaxial position were present near the tip of the leaf; on proceeding basipetally a solenostele with a very small pith was present for a short distance, and below this a

TEXT-FIG. 32. Wound tissue (*w.t.*) round an incision adaxial to a P_1 primordium which developed as a bud, from a longitudinal section through the bud and the shoot apex, which is to the right of the figure. Twenty-nine days after the cut was made. Suberized, necrosed tissue resulting from the incision is stippled. (× 190.)

protostele (Text-figs. 28–31). Similar configurations were observed by Wardlaw (1947). In those plugs which gave rise to parenchymatous outgrowths, the outgrowth itself was devoid of vascular tissue, but a protostele was present in the isolated plug (Text-figs. 29–31).

Bud ontogeny

The ontogeny of the induced buds is characterized by the outgrowth of a group of large prism-shaped cells which divide anticlinally, and by the active division of the underlying cells, vertical growth being greatest at the centre of the meristem; this results ultimately in the formation of the characteristic tetrahedral apical cell (Pl. VI, Figs. 6–9). In the early stages of bud formation anticlinal divisions continue to take place in all the cells of the bud meristem,

including the apical cell (Pl. VI, Fig. 6), and there is no conspicuous enlargement of a single cell as in leaf development. A dome-shaped mass of meristematic tissue is formed, in which the surface layer of cells consists of uniform prism-shaped cells (Pl. VI, Fig. 7). Wardlaw (1946) has shown that buds which develop from detached meristems in *Matteuccia struthiopteris* and *Onoclea sensibilis* possess a similar layer of uniform cells in the early stages of growth before a tetrahedral apical cell is formed. In these induced buds of *Dryopteris aristata*, the formation of a tetrahedral apical cell does not necessarily precede the inception of leaves (Pl. VI, Fig. 8); its formation is apparently the result of particular dispositions of growth in the bud, which are outwardly expressed by the attainment of conical form (Pl. VI, Fig. 9).

Longitudinal sections of isolated buds revealed the presence of a cambiform tissue beneath the cut surfaces (Text-fig. 32). Wound tissue of a similar nature has previously been observed in the more meristematic areas of the petioles of certain ferns and in branches of *Psilotum triquetrum* (Holden, 1912, 1930); it has also been observed in apical tissues of certain angiosperms subjected to similar incisions (Pilkington, 1929; Snow and Snow, 1931).

Discussion

From the foregoing observations and experiments the following principal conclusions can be drawn: (*a*) in *Dryopteris aristata* the determination of a lateral primordium as a leaf normally coincides with the formation of a lenticular apical cell; (*b*) primordia which are isolated from the shoot apex by a wide and deep tangential incision at any time prior to determination are capable of development as buds; (*c*) isolation of a young primordium by deep incisions in close proximity to it may prevent its meristematic growth and result in a parenchymatous outgrowth.

It is clear, therefore, that in this species a primordium which under normal conditions will develop as a leaf is initially capable of development either as a leaf or as a bud, and that a wide and deep adaxial incision can change its prospective fate. Both the depth and the width of this incision are important: it has now been shown that young leaf primordia and presumptive leaf sites do not develop as buds when isolated from the shoot apex by a shallow tangential incision, penetrating only the superficial layer of cells of the apical meristem and leaving the prestelar tissue intact (Wardlaw and Cutter, 1954, 1955); and primordia isolated by a localized deep adaxial incision immediately axillary to the primordium did not develop as buds, whereas a proportion of similar primordia isolated by two deep adaxial incisions which did not meet, leaving a bridge of intact tissue immediately axillary to the primordium, did develop as buds (Wardlaw, 1955*a*, 1955*b*).

Determination, i.e. the fixation of the fate of an organ or tissue, is a process of fundamental importance in development, and is probably indicative of critical metabolic activities. These may relate either to changes in the

localization of important metabolic materials, or to changes in the distribution of growth in tissues, or to both. Establishment of the coincidence of leaf determination in *D. aristata* with a visible histological change, namely the formation of an enlarged, lenticular apical cell (a correlation already suggested by Wardlaw (1947)), not only renders it possible to predict with a high degree of certainty which leaf primordia will be capable of development as shoot buds under the experimental conditions, but also may well prove valuable in future studies of the physiological processes associated with determination. The time in plastochrones required for leaf determination is clearly different in different apices, since in some specimens P_3 is still undetermined; this is correlated with the phyllotactic system and the size of the subtending shoot apex (Cutter, 1955), but other factors may also be involved.

It is not yet known what causes the enlargement of a single centrally situated cell during the ontogeny of a leaf, but it is considered that the configuration of the leaf apical cell may be due to the position of the primordium upon the conical shoot apex and to the stresses resulting from the distribution of growth in that sector of the apex (Wardlaw, 1949*c*, 1955*a*). The occurrence of organs possessing a lenticular apical cell which are of radial symmetry and are capable of indeterminate growth, e.g. the rhizome of *Matteuccia struthiopteris* (Wardlaw, 1949*c*), and of dorsiventral organs with a tetrahedral apical cell, e.g. the rhizome of *Polypodium* and leaves of *Osmunda* and *Todea* (Bower, 1923), suggests that there is no obligate relationship between either dorsiventrality or limited growth and the form of the apical cell *per se*.

There are several possible explanations for the parenchymatization of young primordia when isolated by cuts close to the growth centre. It may be that the small size of the subtending plug of parenchyma precludes the supply of sufficient nutrients or other substances necessary for meristematic growth; older primordia will have a greater capacity to assimilate nutrients and will be able to supplement the materials available in close proximity with supplies drawn through the plug of parenchyma. In addition there may be a diversion of substances necessary for meristematic growth to the wound tissue formed as a result of the cuts (Wardlaw and Cutter, 1954, 1955). The possibility of auxin inactivation at the cut surfaces (Steeves, Morel, and Wetmore, 1953) should also be considered. The proximity of the cuts to the primordium may impose upon it additional stresses, for example as a result of the drying of the exposed tissue, and Wardlaw (1949*d*) has already suggested and provided some evidence which indicates that tensile stress may contribute to the parenchymatous development of meristematic cells of the apical meristem.

The slow establishment of normal spiral phyllotaxis in buds induced by four deep incisions, as compared with that in the majority of those induced by damage to the shoot apex, is of considerable interest and may possibly be correlated with certain anatomical differences. Thus, it is known that the vascular system of buds formed in close proximity to the shoot apex becomes joined to that of the main shoot (Wardlaw, 1943*a*, 1943*b*), whereas that of buds induced by means of four deep incisions apparently never makes

contact with components of the shoot stele. Together with recorded observations upon the inception of phyllotaxis in angiosperm seedlings, this suggests that the availability of nutrients or other metabolites may be a factor in the establishment of phyllotactic systems. Since, however, normal spiral phyllotaxis, albeit of a lower system, is retained in starving apices of *Dryopteris aristata* (Cutter, 1955), this suggestion should be regarded only as a possible basis for further experiment. It may be noted also, as an alternative to the suggested effect of nutrition on phyllotaxis given above, that whereas buds induced by four deep incisions are isolated from the effects of adjacent leaf primordia, buds which are formed on a damaged shoot apex are still subject to the full effects of adjacent primordia.

The observation that no vascular contact is made between primordia isolated by four deep incisions and components of the shoot stele may also be of some importance in assessing the reasons for the eventual retardation or cessation of growth in both isolated leaves and buds. For example, it has been shown above that restriction of the supply of nutrients by undercutting the plug may slow down the inception of leaf primordia on induced buds. It appears, therefore, that while it has been conclusively demonstrated experimentally that isolated meristems which have no vascular connexion with older subjacent tissues can and do derive nutrients through the pith parenchyma (Ball, 1952; Wardlaw, 1945*b*, 1947, 1949*c*, 1950, 1952), such a source of nutrition may become limiting after a time, in the absence of an effective root system. It is known from the work of Wetmore (1950, 1954) and of Sussex and Steeves (1953) that both excised shoot apices and leaf primordia of ferns are capable of continued growth and development in the presence of an adequate source of nutrition.

The importance of the prestelar tissue in morphogenesis is further indicated by the fact that no effect of season or growth rate was observable in the case of experiments with deep incisions, whereas in the case of similar experiments with shallow incisions, in which the prestelar tissue remained intact, the growth rate and general reactivity of the apex was apparently an important factor (Wardlaw and Cutter, 1954, 1955). Seasonal changes in the apex of *D. aristata* are known to include variation in the prominence of the prestelar tissue (Frazer, 1946).

Without greater knowledge of the physiology and biochemistry of the shoot apex it is difficult to reach any adequate interpretation of surgical experiments. The morphological effect of a wide and deep adaxial incision is clear-cut and profound; in *D. aristata* it can cause a primordium which would normally develop as a leaf, an organ of dorsiventral symmetry and rapid but determinate growth, to develop as a bud, i.e. an organ of radial symmetry and potentially unlimited growth. Comparable experiments on some angiosperms have led to the development of a radial leaf under certain conditions (Sussex, 1951; Snow and Snow, 1942, 1954), but buds have not yet been experimentally induced in angiosperms by this means. The effect of such an incision on the physiology of the apex is likely also to be profound, but is at present

not understood. Wardlaw (1955*a*, 1955*b*) has recently pointed out that a wide and deep adaxial incision will interrupt both the basipetal transmission of possible growth-regulating substances or their precursors, and the acropetal flow of nutrients, and will considerably affect the normal regulated growth and metabolism of adjacent tissues. The importance in morphogenesis of the balance between growth-regulating substances and nutrients has already been emphasized by Skoog (1950), and Allsopp (1955), in a consideration of the mechanism of apical dominance, has concluded that both the general nutrient level of the tissues and the relative proportions of growth-promoting and growth-inhibiting substances may be important in correlative growth.

Summary

1. Young leaf primordia of *Dryopteris aristata* were isolated from the shoot apex and (usually) also from adjacent leaf primordia by various systems of deep vertical incisions which severed the prestelar tissue and penetrated into the pith.

2. Leaf primordia isolated from the shoot apex by a wide and deep vertical incision penetrating into the pith at any time prior to the formation of an enlarged, lenticular apical cell were capable of development as solenostelic buds.

3. By inference, in this species the determination of a primordium as a foliar organ normally coincides with the formation of an enlarged, lenticular apical cell.

4. The time between the inception of a primordium and its determination as a leaf is variable in different apices; it may occur during the first plastochrone or as late as the end of the third plastochrone.

5. Isolation of very young leaf primordia by four deep incisions in close proximity to the primordia often resulted in cessation of meristematic growth and the development of parenchymatous swellings.

6. No effect of season of collection or growth rate of the apices was observable on the development of isolated primordia.

7. In the case of isolated primordia which developed as buds, meristematic growth took place over the whole area of the isolated plugs of tissue; in the case of those which developed as leaf primordia meristematic growth was largely restricted to the primordium itself.

8. Neither isolated primordia which developed as buds nor those which developed as leaves underwent marked growth over an extensive period of time; this is attributed to the absence of vascular connexions with the shoot stele.

9. Marking of the isolated primordia with indian ink indicated that the apex of the isolated primordium did give rise to the apex of the bud.

10. The first leaf primordia of the induced buds arose on the longer axis of the isolated plug. Normal spiral phyllotaxis usually was not established until after the formation of about six leaf primordia.

11. A band of uniform prism-shaped cells was present in the developing buds; a tetrahedral apical cell was eventually formed by the oblique division of one of these cells, but its formation did not necessarily precede the inception of leaf primordia on the bud.

12. It is concluded that the effect of a wide and deep adaxial incision upon the development of a primordium may be attributed to its effect on the balance of growth-regulating substances and nutrients, and consequent disturbance of the growth and metabolism of that sector of the apex.

Acknowledgements

I wish to express my thanks to Professor C. W. Wardlaw for suggesting the problem, and for his stimulating guidance and continued encouragement throughout the investigation. I have pleasure also in thanking Mr. E. Ashby and Mr. G. Barker for the photographic illustrations, and the University of St. Andrews for the award of a Berry Scholarship, during the tenure of which this work was carried out.

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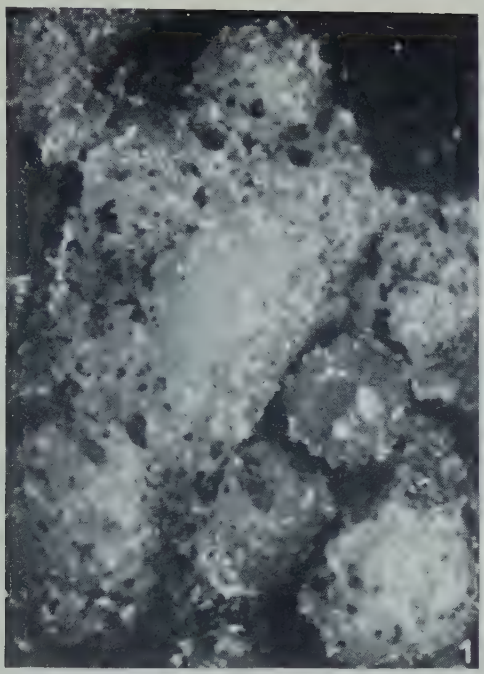
EXPLANATION OF PLATES

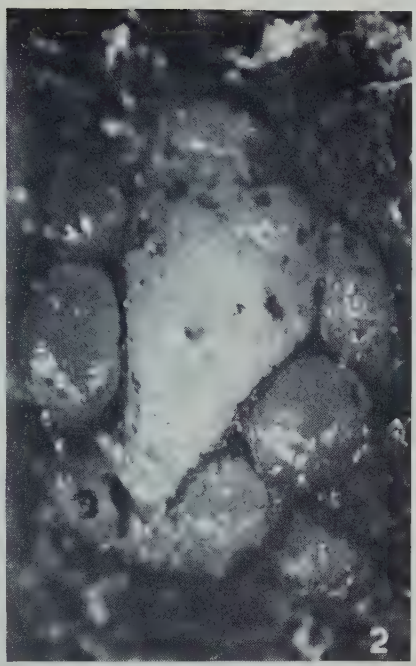
Illustrating Dr. E. G. Cutter's paper on 'The Experimental Induction of Buds from Leaf Primordia in *Dryopteris aristata* Druce'.

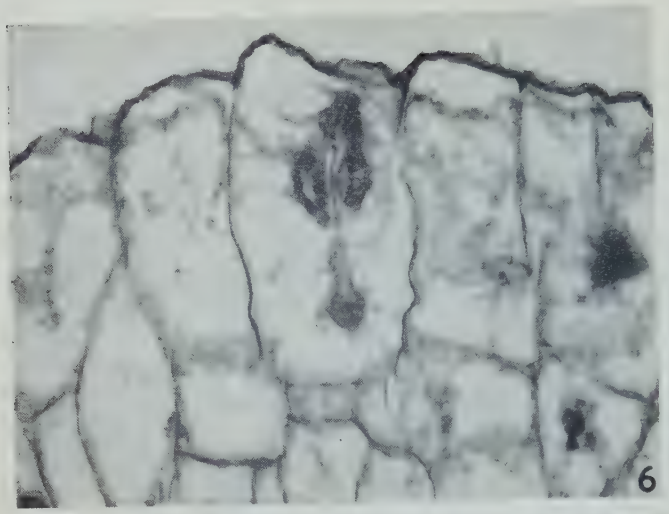
PLATE IV

Fig. 1. Surface view of a specimen 40 days after isolation of P_1 on a large plug of tissue by four deep incisions. A conical bud with three leaf primordia has developed from P_1. Six new primordia have been formed on the shoot apex since the beginning of the experiment. (See Text-fig. 5.) (×15.)

Figs. 2 and 3. Surface views of a specimen in which P_1 and P_2 were isolated on large plugs of tissue by four deep incisions. In Fig. 2, taken 18 days after isolation, large dome-shaped swellings have developed from P_1 and P_2; in Fig. 3, taken 35 days after isolation, these have developed as buds, each with three leaf primordia. (See Text-figs. 7 and 8.) (×20.)



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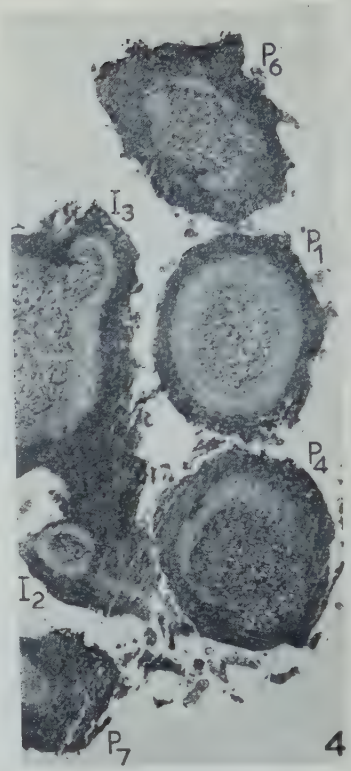
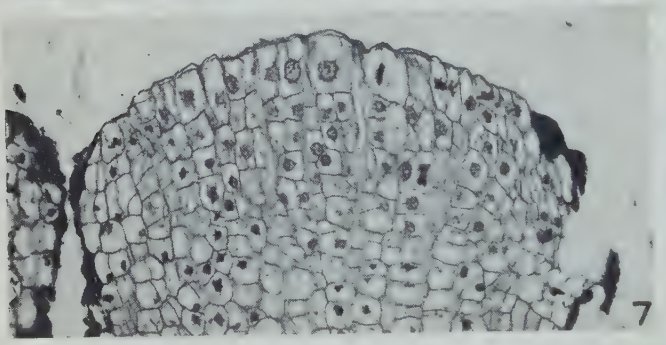

P6
I3
P1
P4
I2
P7
4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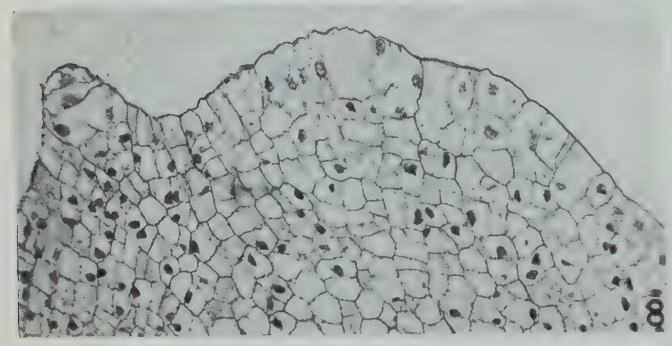
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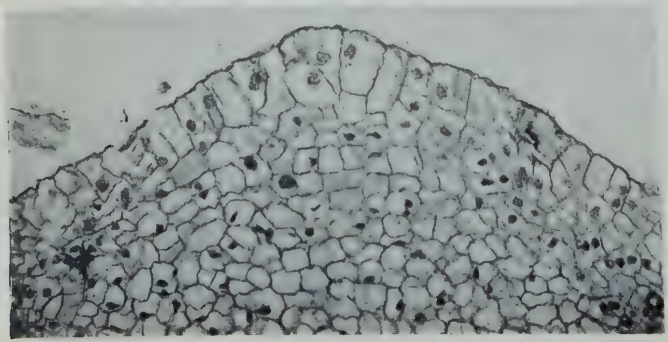
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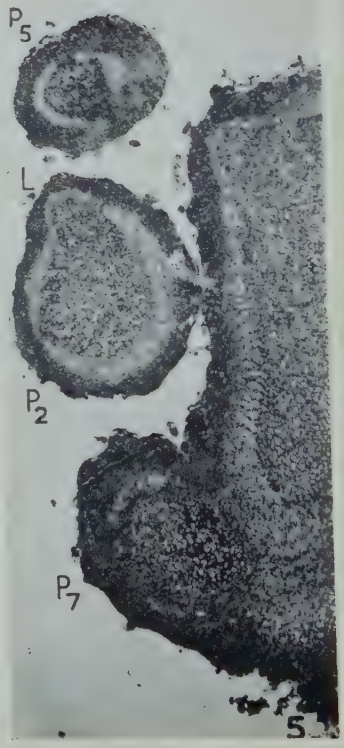
P5
L
P2
P7
5

PLATE V

Figs. 4 and 5. Transverse sections of the apex illustrated in Pl. V, Figs. 2 and 3, and Text-figs. 7 and 8, in which P_1 and P_2 developed as buds as a result of isolation by four deep incisions. In Fig. 4 the solenostelic vascular structure of the bud which developed from P_1 is clearly shown; Fig. 5, from a more basipetal section, shows the solenostelic structure of the P_2 bud, in which a leaf primordium (L) is present on the left. Note the continuous adaxial strand of prestelar tissue in P_4, which has been partially isolated by the cuts (Fig. 4). (×20.)

Figs. 6–9. Longitudinal sections of induced buds in various stages of development.

Fig. 6. Anticlinal division of the central cell of the meristem in a P_2 plug 3 days after isolation of P_2. (×500.)

Fig. 7. Longitudinal section of a P_1 plug 13 days after isolation of P_1, showing the domed shape of the induced growth. The apical meristem consists of a band of uniform prism-shaped cells. (×125.)

Fig. 8. An induced bud with its first leaf primordium, 26 days after isolation of P_1. A tetrahedral apical cell is not yet present in the bud apex. (×125.)

Fig. 9. An induced bud, 38 days after isolation of P_1, showing the formation of the tetrahedral apical cell by an oblique division of one of the prism-shaped cells of the apical meristem. Four leaf primordia were present on the bud. (×125.)

The Development of the Runner Bean Leaf with Special Reference to the Relation between the Sizes of the Lamina and of the Petiolar Xylem[1]

III. The Development of the Leaf under Various Conditions

BY

D. J. B. WHITE

(*Department of Botany, University College, London*)

With four Figures in the Text

ABSTRACT

The development of the first pair of leaves of the Runner Bean (*Phaseolus multiflorus* Willd.) under various conditions is described.

1. Under damp conditions and also when one leaf of a pair was covered with a black paper envelope, the *rate* of development was altered but no significant changes in the relative development of the parts of the leaf occurred.

2. Removal of a portion of the lamina at an early stage of development resulted, at maturity, in a leaf having relatively *less* petiolar xylem, although the ratio xylem area/lamina area was slightly higher. This treatment also delayed a 'sudden' increase in area of the vessels which was seen in normal plants.

3. An attempt was made to estimate the transporting power of the petiolar xylem and to compare it with the lamina area. The data suggest that the maximum possible area of the lamina at any stage in development may be determined by the transporting power of the xylem, but that the attainment of this maximum possible area may be prevented by external factors as is seen in some of the experimental results presented.

IN a previous paper in this series (White, 1954*b*) the normal development of the bean leaf was described on the basis of measurements made on a group of seedlings ('control group') grown without any special treatment. Alongside these seedlings were grown three other groups which received different treatments with the idea of attempting to modify the relative development of the different parts of the leaf. The development of these plants was compared with that of the control group and from the results certain conclusions may be drawn. The treatments given were as follows:

1. Fifty plants were covered with belljars so that they were growing in a damp atmosphere. During this experiment there was one abnormally hot period and the plants became overheated. The effect of this was to inhibit lamina extension for two days. These plants are referred to as the 'damp-air group'.

2. In each of another 50 plants one of the pair of leaves was covered by a black paper envelope fixed by two paper clips. The development of the

[1] Part of a thesis approved for the degree of Ph.D. of the University of London.

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covered and uncovered leaves was followed separately. The results for this group ('covered group') are subdivided

P_1—Normal leaf;
P_2—Covered leaf.

3. In each of another 50 plants a portion of each lamina was amputated at right-angles to the mid-rib. The reason for amputating at right-angles rather than parallel to the mid-rib was to eliminate, as much as possible, tearing of the lamina during subsequent growth. These plants are referred to as the 'amputated group'.

Five plants were removed for measurement from each of the above three groups and from the control group[1] on July 22, 24, 26, 28, 30, and August 3, and in the case of the covered groups on August 10. The plants removed on each occasion are referred to as Sample 1, 2, &c.

In each instance

(1) the length and area of the petiole;
(2) the area of the petiolar xylem and the number of vessels; and
(3) the length, breadth, and area of the lamina

were determined by the methods previously used (White, 1954*a*). The plants after receiving the various treatments on July 20 grew healthily.

On the day on which the treatments were started (i.e. July 20) five plants from the control group were measured. This set of five plants, referred to as the 'Initial Sample', forms the starting-point for all the groups.

The average values for the samples of each group of plants are given in Tables I–IV in the appendix.

The Effects of the Various Treatments on Development

1. The development of the leaves of the plants in damp air, and the normal leaves (P_1) of the plants of the 'covered group', did not differ significantly, except in *rate*, from the development of the leaves of the control groups. It is therefore unnecessary to discuss the results for these groups.

2. *The 'Covered-leaf' group*

As far as external appearances went, this leaf made some growth, then began to turn yellow, shrink, and finally withered. The tenth day was the last on which it was possible to make any measurements. The 'covered' leaves had fallen within the next day or so.

Growth of the lamina and petiole. The lamina grew slightly during the first four days. It increased, for example, in area from 6·0 sq. cm. to 7·5 cm. After this time shrinkage took place.

The early growth of the petiole was normal, but it then slowed down and by the end of the first week a certain amount of shrinkage had taken place.

Xylem development in the petiole. Xylem production was affected by covering the leaf but not to the same extent as was the growth of the lamina. The

[1] Detailed results for the control group were given in the previous paper (White, 1954*b*).

xylem increased in *area* up to the fourth day, after which it decreased. The decrease is firstly due to the loss of protoxylem vessels which are not replaced, and in the later stages to the shrinkage of the petiole previously referred to. The *number* of vessels actually continued to increase up to the sixth day, after which it fell.

The development of the average area of the vessels is normal. There is an increase in area taking place in the first two days and thereafter the average area of the vessels fluctuates about a mean of $10{\cdot}3 \times 10^{-4}$ sq. cm. Thus covering the leaf had no significant effect upon the size of the vessels produced.

The relationship between xylem area (X) *and lamina area* (L). The ratio X/L

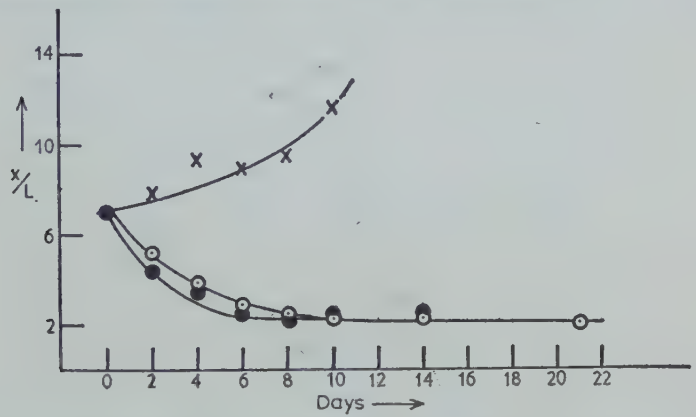

FIG. 1. Ratio X/L for control leaves (dots), normal leaves (circles), and covered leaves (crosses).

fell slightly during the first two days, and thereafter rose continuously, and was at its maximum value when the last measurements were made, i.e. on the tenth day (Fig. 1). This graph brings out the difference in behaviour of the xylem and lamina following covering. Although extension of the lamina was, to all intents and purposes prevented, xylem production was not immediately affected.

Relationship between vessel number (V) *and lamina area* (L). As might be expected from what has been written above, the ratio V/L increases steadily and, like the ratio X/L, has its highest value on the tenth day, the last occasion on which it was possible to make observations on these leaves.

It is clear that vessel production continues for a time at any rate, even although the lamina is making little or no growth.

Summarizing the effects of covering one leaf:

The '*normal*' (i.e. uncovered) leaf did not differ significantly from the control plants.

'*Covered*' leaf: the lamina was most affected, very little further growth was made. The petiole and xylem continued to grow for some 4–6 days and during this time their development was normal.

3. *'Amputated-leaf' group*

Lamina. The growth in area of the lamina was normal. The growth curve is shown in Fig. 2. The final size of the lamina (40 sq. cm.) was about two-thirds the final size of the control plants. Half the final size had been attained by about the seventh day, which was somewhat later than in the case of the control plants.

Petiole. Growth in length of the petiole was unaffected during the first two

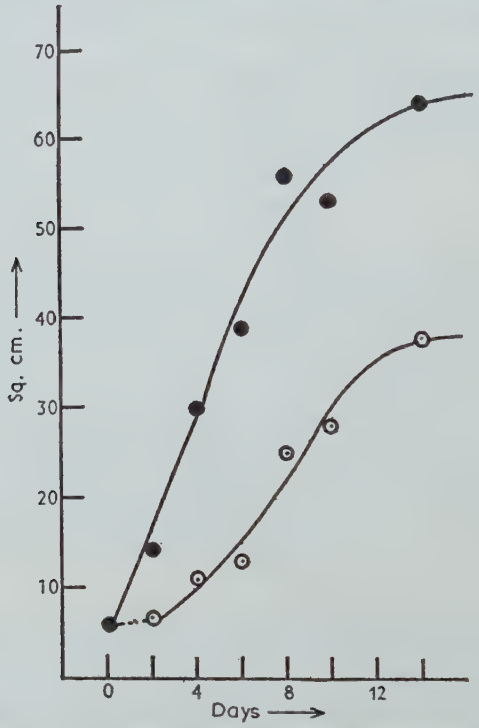

FIG. 2. Growth curves for lamina area. Control leaves (dots), and amputated leaves (circles).

days following amputation. It then became slower, and at maturity the petioles of the amputated leaves were slightly shorter than the controls.

Growth in cross-sectional area of the petiole was affected sooner and to a greater extent than was its growth in length. The petiole continued to increase its cross-sectional area after its growth in length had ceased.

Xylem development in the petiole. This was similar to that for the control plants, but less xylem was present at all time stages. By comparison with both the control plants and the 'normal' leaf of the covered, it is clear that amputation of part of the lamina at an early stage in development results in less xylem (as measured by its cross-sectional area) being formed.

Vessel number increased fairly rapidly during the two days following amputation. The rate of increase then dropped somewhat compared to the

control plants. The final vessel number was not greatly affected by amputation (cf. xylem area). At maturity there were 246 vessels as compared to 274 for the normal leaves, and 309 for the controls.

The vessels did NOT show the 'sudden' increase in average area during the first two days, such as was seen in the other groups of plants investigated. The increase took place later, during the period 2nd–4th days. Thereafter the average area fluctuated about a mean of $10{\cdot}1 \times 10^{-4}$ sq. cm. (Fig. 3).

It was pointed out for the control plants that the increase in vessel area occurred appreciably before the lamina had reached half its final size. In the amputated plants in which increase in average vessel area was delayed, the lamina does not reach half its final size until later (compare Figs. 2 and 3).

The relationship between xylem area (X) *and lamina area* (L). Following

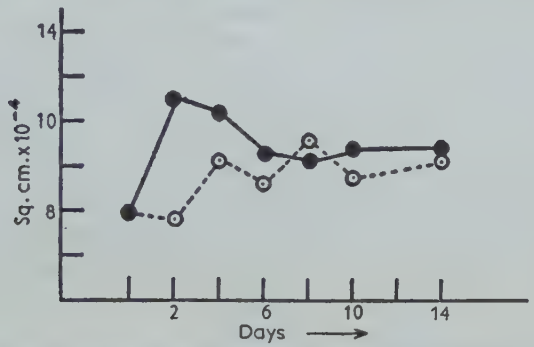

FIG. 3. Average area of vessels of control leaves (dots) and amputated leaves (circles).

amputation the ratio X/L rises, since a portion of the lamina has been removed, and then it falls, at first more slowly than in the controls. At maturity the value of this ratio is rather higher than in the other groups of plants; i.e. at maturity the leaf has slightly more xylem per unit area of lamina than is the case in the uncut leaves.

Amputating a portion of the lamina at an early stage of development results in less xylem being formed, so that the xylem is brought approximately into the 'normal' relationship with the lamina, even although the 'normal' relationship is disturbed at the time the amputation is performed.

The relationship between vessel number (V) *and lamina area* (L). The ratio V/L rises very sharply following the removal of a portion of the lamina (vessel production was not affected during this time), and then falls. The value of the ratio V/L at maturity was rather higher than in the control plants (6·5 as against 5·0).

A direct comparison of vessel number with lamina area shows that for any particular vessel number the lamina has a smaller area than a leaf of the control plants with a similar number of vessels.

Summarizing the effect of this treatment. Amputating part of the lamina at an early stage of development does not affect the nature of the growth relationships between parts of the leaf. At maturity the leaf has less xylem

(as measured by its area cross-section) than the normal leaf, and the ratio X/L is slightly higher. The number of vessels is not so greatly affected. The removal of part of the lamina delays the 'sudden' expansion of the vessels seen in the normal plants.

Discussion of the Xylem–Lamina Relationships

The main points in the relationship may be summarized:

1. The ratio X/L decreases with development: the value of the ratio is minimal at maturity.
2. The effect of some changes in external conditions is to alter the *rate* at which the minimum value is reached.
3. Growing the leaf under damp conditions has little effect upon the final area of the xylem produced in the petiole.
4. When the lamina is artificially reduced in area by amputation of part of it at an early stage, the xylem, although reduced in area, is not reduced to the same extent as the lamina area. The ratio X/L at maturity is higher than for the other groups of plants.
5. Inspection of the data given in Table IV in the Appendix shows that amputation of part of the lamina reduces the number of vessels produced to about 80 per cent. of the controls, and the increase in average vessel size is delayed.

The diminishing nature of the relationship as shown by the decrease in the ratio X/L suggests, as was pointed out earlier, that the amount of the xylem produced is not necessarily related to the water requirements of the leaf. This view is supported by the absence of effect upon the xylem when the leaf is grown under damp conditions, and by the results obtained for the amputated plants.

However, the transporting power of the xylem is not measured by the xylem area. An attempt has therefore been made (as far as the data permit) to estimate the transporting power of the xylem at different stages of development.

The volume V of liquid delivered in time t through a tube of length l and radius r is given by the equation

$$V = \frac{pr^4t}{8lv}$$

where p = the pressure difference between the ends of the tube, and v = the coefficient of viscosity of the liquid.

It is necessary to assume that p, t, l, and v are the same for the various leaves, then for comparative purposes the transporting power may be represented by:

The number of vessels $(V) \times$ (average radius of vessels)^4.

In the particular instance under consideration the number of vessels (V) is known and r may be obtained from the average area of the vessels.

In Fig. 4 (top) the estimated transporting power of the xylem for the control leaves (data presented in previous paper), and the normal leaves of the covered plants, has been plotted against the lamina area. This gives a good

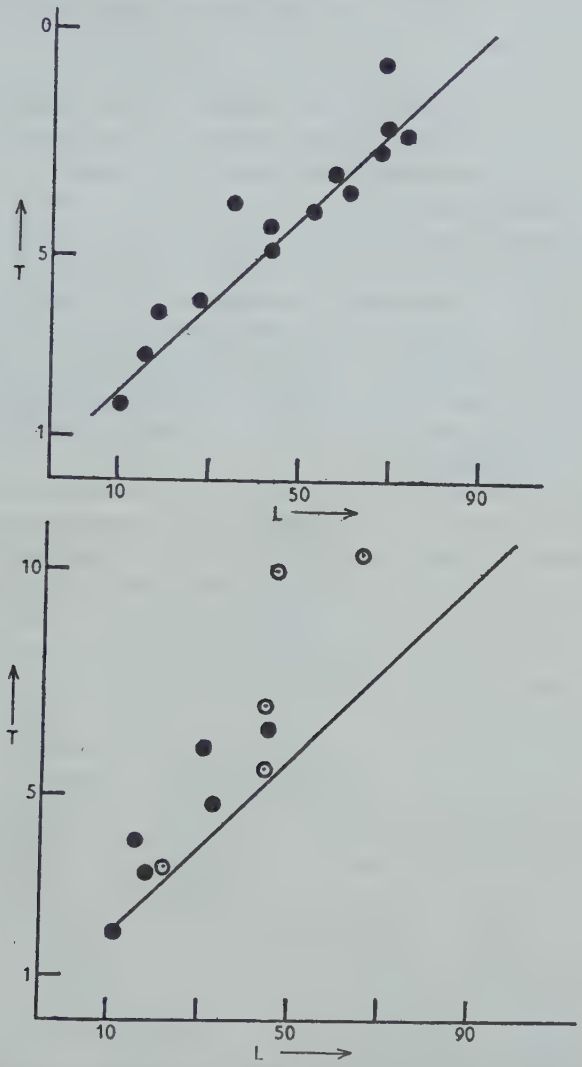

FIG. 4. Estimated transporting power (T) of the petiolar xylem plotted against lamina area (L) for control and normal leaves (top); and below, for damp air (circles) and amputated leaves (dots). The line for the control and normal leaves has been inserted in the lower graph.

approximation to a straight line, as drawn, the two high points being ignored.

Fig. 4 (bottom) shows the results for damp air and amputated leaves with the line for the control and normal leaves inserted. The first two sets of observations for the damp air leaves fall on this line, the later ones do not,

i.e. up to the time lamina expansion was stopped (Table I) the relationship between the transporting power of the xylem and the lamina area is the same as for the control and normal leaves.

The data for the amputated plants do not fall upon this line. They have, of course, smaller laminae, artificially caused, although the petioles possess a more normal transporting power.

The data suggest, as an hypothesis, that the transporting power of the petiolar xylem may determine the maximum possible area of the lamina at any stage in development; but that the attainment of the maximum possible area by the lamina may be prevented by external factors, as for example, as happened in the treated plants.

The average area of the vessels increases suddenly during development, shortly after the initial expansion of the lamina and well before the lamina is half grown. This may possibly be due to the diffusion from the lamina at this stage (or diffusion in greater quantity at this stage) of some substance or substances which influence the degree of extension of the vessels. Thus, when a portion of the lamina is removed at an early stage, the increase in average vessel area is delayed.

At the stage this investigation has taken for its starting-point both lamina and petiole have begun their development. It leaves untouched the very fundamental problem of the relationship between the formation and development of a foliar primordium and the procambial strand associated with it. The general consensus of opinion at the present time seems to be that the procambial strand develops acropetally from the older procambial tissue or vascular tissue and is present at the base of a foliar primordium at a very early stage. (See Esau 1943.)

There appears to be no evidence available as to what determines either the size of the primordium or of its attendant procambial strand.

But once the primordium and procambial strand are developing the subsequent development of the leaf must be viewed in terms of reciprocal effects between petiole and lamina. Thus the initial expansion of the lamina is associated with a 'sudden' increase in the average area of the vessels of the petiole. When a portion of the lamina is removed at an early stage the increase in vessel size is delayed. This clearly points to an effect of the lamina on xylem development in the petiole.

On the other hand, the maximum area which may be attained by the lamina at any stage can apparently be related to the transporting power of the xylem, although the attainment of this maximum area may be prevented by the operation of certain external factors.

The vascular supply both in terms of its transporting power and its distribution is clearly a factor of some importance in determining leaf form and size. It has not perhaps received as much attention as it merits, although it was considered by Pearsall and Hanby (1926) in their study of the factors affecting the development and form of leaves, particularly the palmate leaves of some dicotyledons.

Acknowledgements

I am greatly indebted to the late Professor T. G. Hill, Emeritus Professor of Plant Physiology in the University of London, for suggesting the problem; to Professor W. H. Pearsall for his help and especially for several profitable discussions; and to Mr. N. W. Please of the Department of Statistics, University College, London, who examined my data statistically for me.

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Appendix

Table I

Measurements for the leaves in damp air

Sample	Lamina area in sq. cm. (L)	Xylem area in sq. cm. × 500 (X)	Number of vessels (V)	Average area of vessels sq. cm. × 10^{-4}	Ratio X/L	Ratio V/L
1	17·10	63·1	117	11·4	3·9	7·1
2	38·65	99·9	182	10·8	2·53	4·87
3	[1]39·19	119·7	208	11·8	3·21	5·53
4	41·28	154·2	238	13·2	3·81	5·84
5	59·52	170·9	286	12·3	2·31	5·12

	Lamina			Petiole		
Sample	length 'l' cm.	breadth 'b' cm.	Ratio l/b	length cm.	area P sq. cm.	X as percentage of P
1	5·04	4·34	1·17	3·6	2·01	6·3
2	7·7	6·5	1·2	5·7	2·65	7·51
3	[1]7·49	6·47	1·15	6·0	2·43	9·90
4	8·07	6·83	1·19	6·8	2·78	11·58
5	9·6	8·0	1·20	6·6	3·26	10·49

[1] Lamina extension inhibited. See p. 167.

Table II

Measurements for normal leaves of a pair of which the other was covered (see Table III)

Sample	Lamina area in sq. cm. (L)	Xylem area in sq. cm. × 500 (X)	Number of vessels (V)	Average area of vessels sq. cm. × 10^{-4}	Ratio X/L	Ratio V/L
1	12·11	57·0	125	9·5	5·22	12·61
2	23·33	86·17	190	9·1	3·85	8·76
3	37·72	105·1	196	8·6	2·9	5·44
4	47·55	114·65	221	10·5	2·53	4·86
5	62·83	138·5	254	11·0	2·26	4·16
6	64·0	146·3	274	10·9	2·29	4·37
7	67·47	140	258	10·9	2·09	3·87

	Lamina			Petiole		
Sample	length 'l' cm.	breadth 'b' cm.	Ratio l/b	length cm.	area P sq. cm.	X as percentage of P
1	4·3	3·6	1·26	2·9	1·95	5·98
2	6·2	5·0	1·24	4·4	2·61	6·62
3	7·54	6·36	1·20	5·3	3·07	6·87
4	7·98	6·94	1·17	6·0	3·05	7·72
5	8·82	7·88	1·12	6·5	3·28	8·57
6	9·3	8·8	1·12	7·0	3·70	8·04
7	9·42	8·38	1·13	7·1	3·64	7·89

Table III

Measurements for covered leaves (cf. Table II)

Sample	Lamina area in sq. cm. (L)	Xylem area in sq. cm. × 500 (X)	Number of vessels (V)	Average area of vessels sq. cm. × 10^{-4}	Ratio X/L	Ratio V/L
1	6·23	49·9	96	10·3	7·89	15·99
2	7·47	63·52	133	10·5	9·33	18·73
3	6·95	62·14	144	8·6	8·9	20·8
4	7·27	66·26	122	11·4	9·52	16·95
5	5·79	64·45	122	10·6	11·6	21·6

	Lamina			Petiole		
Sample	length 'l' cm.	breadth 'b' cm.	Ratio l/b	length cm.	area P sq. cm.	X as percentage of P
1	3·3	2·6	1·27	2·9	1·61	6·16
2	3·6	2·8	1·36	3·9	2·08	6·62
3	3·32	2·68	1·26	3·8	2·01	6·25
4	3·36	2·82	1·19	4·1	1·92	7·06
5	3·2	2·38	1·36	4·2	1·78	7·26

TABLE IV

Measurements for leaves reduced to about half the original area by amputation

Sample	Lamina area in sq. cm. (L)	Xylem area in sq. cm. × 500 (X)	Number of vessels (V)	Average area of vessels sq. cm. × 10^{-4}	Ratio X/L	Ratio X/L
1	6·64	50·84	134	7·6	7·8	20·8
2	10·87	76·91	151	10·3	7·43	14·88
3	13·21	70·41	153	9·2	5·29	11·81
4	25·46	110·81	203	11·2	4·49	8·16
5	27·76	100·02	213	9·5	3·64	7·87
6	37·98	126·06	246	10·3	3·32	6·52

	Lamina			Petiole		
Sample	length 'l' cm.	breadth 'b' cm.	Ratio l/b	length cm.	area P sq. cm.	X as percentage of P
1	1·7	3·6	0·48	2·5	1·49	6·9
2	1·8	4·9	0·37	4·2	1·83	8·45
3	2·1	5·1	0·42	4·7	2·09	6·86
4	2·6	7·2	0·36	5·3	2·33	9·58
5	3·1	7·3	0·43	5·2	2·67	7·71
6	2·9	9·0	0·33	5·4	3·20	8·02

Studies in the Physiology of Parasitism

XXII. The Production of Pectolytic Enzymes by *Pythium de Baryanum* Hesse

BY

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With five Figures in the Text

ABSTRACT

The incorporation of sodium chloride in a synthetic medium stimulated the pectolytic activity of cultures of *Pythium de Baryanum*. The Cl^- ion appeared to be mainly responsible for this effect; on the other hand, presence of the Ca^{++} ion depressed enzymic activity.

Glucose, fructose, and mannose were about equally suitable for growth and enzyme production. Sucrose, if used as sole carbohydrate source, gave good mycelial growth but poor enzyme production, but if a small proportion was replaced by glucose, enzyme production was as good as on glucose itself. Galactose gave very poor growth and negligible enzyme production.

For optimum production of pectolytic enzyme, glucose (or fructose or mannose) requires to be autoclaved in a somewhat alkaline medium—very conveniently with the K_2HPO_4 or K_3PO_4 of the nutrient medium. A yellow to brown coloration (due to caramelization) is produced in the process, but the stimulating factor is not bound up with the colouring substance. The same stimulating effect on enzyme production was obtained by adding to the nutrient medium a small quantity of glucose which had been dry heated at 150° C. for 20 minutes. Chromatographic analysis suggested that the stimulating substance was probably glyceraldehyde, though it is not excluded that other breakdown products of sugars may also play a part.

A. INTRODUCTION

CERTAIN parasites such as *Botrytis cinerea* and *Bacterium aroideae* produce pectolytic enzymes on a great variety of media, natural or synthetic, and over a wide range of conditions. With *Pythium de Baryanum* it is otherwise, so that earlier workers—Chona (1932), Menon (1934), and Fernando (1937)—who studied the physiology of this fungus were limited to preparing active extracts from parasitized potato tissue. Later, Ashour (1949, 1954) developed a synthetic medium, based upon the crude chemical composition of potato tubers, which was suitable for enzyme production, the notable feature of this medium being that any change, upwards or downwards, in concentration of its constituents caused a pronounced fall-off in secretion of enzyme.

Damle (1952), working with Ashour's synthetic medium, found that active secretion of enzyme was not obtained unless the glucose constituent was

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autoclaved with phosphate (K_3PO_4 or K_2HPO_4). Autoclaving with acid phosphate (KH_2PO_4) was ineffective, even when the medium was subsequently adjusted to a pH favourable for the growth of the fungus. Incidentally Damle noted that when the medium was prepared in such a way that the carbohydrate (glucose or fructose) was autoclaved with tribasic or dibasic phosphate (with or without the other constituents), it developed a yellow to brown colour. Hence it came about that this coloration was a sign that the medium was suitable for vigorous secretion of enzyme.

The effect of heating together phosphate and sugar on the growth of filamentous fungi has not hitherto been reported, but there are a few references concerning a similar phenomenon in studies of bacteria and yeasts. Fulmer and Huesselmann (1927) state that when glucose and K_2HPO_4 are heated under pressure, substances are formed which stimulate the growth of yeast. Later Fulmer, Williams, and Werkman (1931) report that under these conditions bacterial growth substances are formed. The darkening effect was interpreted to mean that some caramelization had taken place, but tests with a commercial caramel of unknown origin and with glucose which had been caramelized (by heating at 200° C. for 20 minutes) gave only slight stimulation. Conversely when caramelized medium was decolorized by active charcoal, it retained the stimulating power. They concluded that the stimulation was not due to caramelization as such but to some substance produced at the same time as the caramel.

The further examination of this problem, viz. the rather specialized behaviour of *Pythium de Baryanum* in the matter of pectolytic enzyme secretion, is the topic of this paper.

B. General Materials and Methods

Stock cultures of *P. de Baryanum* were grown on potato dextrose agar (20 per cent. potato, 2 per cent. glucose, 2 per cent. agar) at room temperature and renewed at monthly intervals.

For preparation of enzyme solutions 15 ml. of medium were placed in flat medicine bottles of 12 fluid oz. capacity, sterilized and inoculated each with 10 discs (4 mm. diam.) cut with a cork-borer from the growing edge of a 2–3-day-old culture on 0·2 per cent. glucose agar. The bottles were then stacked on their flat sides and incubated at 25° for 5 days, preliminary experiments having shown that the peak of enzymic activity was reached in 4–5 days, after which it fell off.

Most of the work was carried out with synthetic media based upon Ashour's formula, which was:

25 g. starch, 5 g. glucose, 4·5 g. bacteriological peptone, 4·5 g. asparagine, 3·4 g. K_3PO_4, 1·9 g. $MgSO_4,7H_2O$; all of analar grade, and made up to 1 litre with distilled water. To a less extent various potato decoctions were also used; in a few cases enzymic preparations were made from the tissue of potato tubers inoculated with the fungus.

At the end of the incubation period the fungal mats were filtered off,

washed, dried, and weighed on a Joly spring balance. The filtrates were tested for pectolytic activity viscometrically, and by the maceration method with standard discs of potato tissue. When it was required, enzyme preparations were kept for long periods at −20° C.; for periods of not more than one week they were kept at 4° C. under toluene, without appreciable loss of activity.

pH changes were followed colorimetrically or by use of a pH meter, or by both methods.

C. Composition of Synthetic Medium as Affecting Enzyme Production

It was soon found that Ashour's medium could be simplified by replacing its content of 2·5 per cent. starch by the same amount of glucose. A medium containing 3 per cent. glucose, instead of the mixed carbohydrates of Ashour's medium, gave equal mycelial growth and equal enzymic activity in the filtrate.

A marked improvement was also obtained by adding a small amount of sodium chloride to the medium. This effect was detectable at a concentration as low as 0·001 per cent. and reached its maximum at about 0·1 per cent. NaCl. The magnitude of the effect is illustrated by the following figures which give the times required for the maceration of potato discs in the respective filtrates:

Culture medium without NaCl	90 min.
„ „ with 0·1% NaCl	25 „

In twelve experiments in which the above comparison was made, the average times for maceration were 80 and 24 minutes respectively, the significance of the difference being much greater than $P = 0{\cdot}01$.

The effect is also illustrated in Fig. 1, which records the fall in viscosity of a 0·5 per cent. citrus pectin solution in presence of culture filtrates. The reactions were carried out at 25° C. and in the presence of Sörensen's phosphate buffer at pH 8·3.

The beneficial effect of 0·1 per cent. NaCl was equalled by the same concentrations of NH_4Cl, KCl, and $MgCl_2$, thus suggesting the importance of the Cl^- ion. On the other hand, some improvement was also shown by addition of 0·05–0·1 per cent. NaOH, $NaNO_3$, Na_2CO_3, or Na_3PO_4. Finally, in presence of 0·1 per cent. $CaCl_2$, enzyme secretion was less than in the control medium, so that the presence of Ca^{++} more than counteracted the stimulating action of the associated Cl^-.

On the basis of these preliminary observations the standard medium adopted was that of Ashour, modified by using 3 per cent. glucose as sole source of carbohydrate and by the addition of 0·1 per cent. NaCl. With this medium a number of Ashour's and Damle's experiments were repeated, with substantial confirmation of their claims that any material alteration, upwards or downwards, of the carbon or nitrogen constituents led to a marked

reduction of enzymic activity. It was also shown that replacement of peptone or asparagine by equivalent amounts, in terms of nitrogen, of such compounds as ammonium tartrate, chloride or nitrate, potassium or sodium nitrate gave

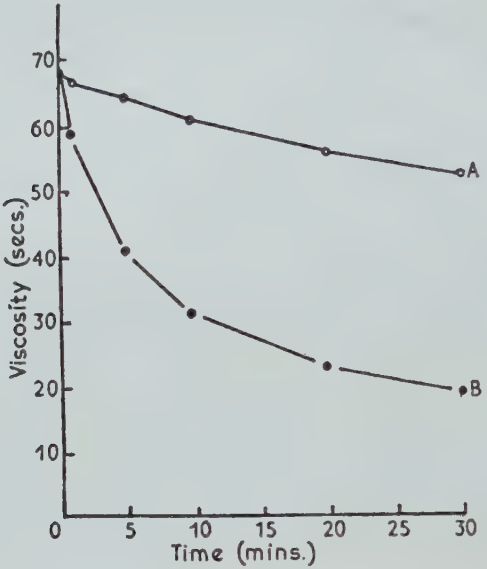

FIG. 1. Effect of sodium chloride on production of pectolytic enzyme by *P. de Baryanum*. A. Ashour's medium. B. The same with 0·1% NaCl present.

reduced enzymic secretion, even in cases where such substitution gave enhanced mycelial growth.

TABLE I

Effect of Concentration of Glucose on Enzyme Production and Mycelial Growth

Medium with:	Init. pH	Final pH	R.T.[1] in mins.	Dry wt.[2] of myc.
5·0% glucose	7·3	8·3	65	3·0
4·0% ,,	7·4	8·3	35	2·4
3·0% ,,	7·5	8·3	22	2·0
2·0% ,,	7·8	8·1	100	1·3
1·0% ,,	8·0	8·0	210	0·8
0·5% ,,	8·1	7·9	5½ hrs.	0·7

[1] R.T. = Reaction Time, i.e. time for maceration of standard potato discs.
[2] In Joly balance units; 15 such units = 1 g. Similarly in other Tables.

The effect of the carbohydrate constituent was examined in some detail. Table I shows the effect of a range of glucose concentrations on growth and enzyme production, the other constituents being as in the modified standard medium.

The most active enzyme secretion was found to be on a medium with 3 per cent. glucose. Variations, upwards or downwards, markedly reduced

activity. The secretion of enzyme did not run parallel to the amount of mycelial growth, which increased steadily with the glucose concentration.

TABLE II

Effect of Different Hexoses on Enzyme Production and Mycelial Growth

Medium with:	Init. pH	Final pH	R.T. in mins.	Dry wt. of myc.
3% glucose . .	7·5	8·2	22	2·0
3% fructose .	7·3	8·1	20	2·7
3% mannose .	7·5	7·9	25	2·4
3% galactose .	7·5	7·6	24 hrs.	0·3

In Table II, four hexose sugars are compared as sources of carbon for enzyme production.

Glucose, fructose, and mannose are more or less equivalent for enzyme

TABLE III

Effect of Glucose and Sucrose on Enzyme Production and Mycelial Growth

Medium with:	Init. pH	Final pH	R.T. in mins.	Dry wt. of myc.
3·0% glucose	7·4	8·1	25	2·1
3·0% sucrose	8·0	8·2	80	2·9
2·5% sucrose+0·5% glucose . .	7·9	8·2	27	2·3

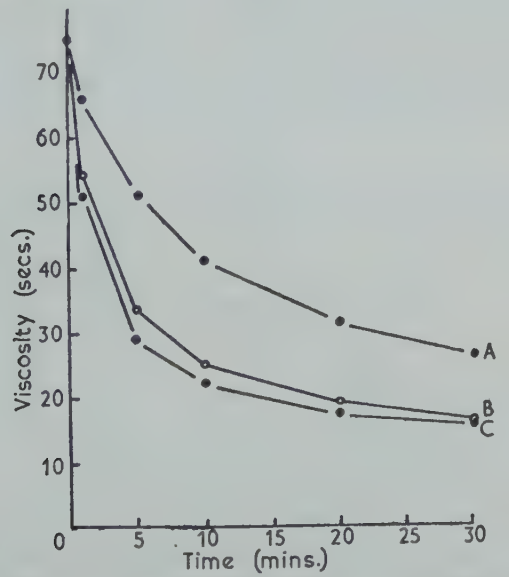

FIG. 2. Production of pectolytic enzyme by *P. de Baryanum* in culture media containing: A, 3% sucrose, B, 3% glucose; C, 0·5% glucose+2·5% sucrose.

production and growth, a result which is probably referable to their giving the same enolic form by hydroxyl migration. On the other hand, galactose has a different configuration, and this may be the reason for its unsuitability.

With all four sugars the colour of the medium changed to yellowish brown during heat sterilization.

The behaviour of glucose and sucrose, separately and together, is set out in Table III.

Sucrose, used as sole carbohydrate source, behaved like starch in giving weak enzyme solutions, and this in spite of more vigorous growth. It is, however, a suitable form of carbohydrate if a proportion of glucose is also present. No yellow colour is formed when the medium containing sucrose only is autoclaved.

The preparations recorded in Table III were also tested viscometrically (Fig. 2) with results which follow the same course as those obtained by the maceration method.

D. Method of Heat Sterilization as Affecting Enzyme Production

Damle's claim with reference to the method of sterilization was fully confirmed in a series of five experiments, the results of which are set out in Table IV. Ashour's medium (see p. 180) was used and the following three methods of sterilization adopted:

A. All constituents together.
B. $MgSO_4$ separately from remainder.
C. K_3PO_4 ,, ,, ,,

A, B, C of Table IV relate to Ashour's medium unmodified; A′, B′, C′ to the same medium with 0·1 per cent. NaCl added.

Table IV

Effect of Method of Autoclaving on Enzymic Activity

Medium	Colour after autoclaving	R.T. (av. of 5 experiments) in mins.
A	Light yellow with ppte.	92±3·8
B	Yellow	51±5·3
C	No change	110±3·2
A′	as A	34±3·7
B′	as B	25±1·6
C′	as C	55±5·9

Statistical analysis: A–B, $P < 0{\cdot}01$ A′–B′, non signif.
C–B, ,, $< 0{\cdot}01$ C′–B′, $P < 0{\cdot}01$
C–A, ,, 0·05–0·01 C′–A′, ,, 0·05–0·01

In Table V the writer's modification of Ashour's medium was used, i.e. the carbohydrate was 3 per cent. glucose only, and 0·1 per cent. NaCl was added. In all the preparations the magnesium sulphate was autoclaved separately. The remainder of the medium was sterilized in a variety of ways, viz. by passing through a Seitz filter, by steaming for 30 minutes on three successive days, by autoclaving at 5 lb. pressure for 20 minutes on two successive days, or by autoclaving once at 10, 15, or 20 lb. pressure for 20 minutes.

The low activity given by the first treatment in Table V shows that heating is required, but a wide range of heat treatments gives the same result. In the last treatment there is an indication of over-heating, as shown by a reduction in growth and in enzymic activity and by the development of greater acidity. The greater enzymic activity obtained by a suitable degree of heating may be accompanied by somewhat greater mycelial growth (as in Table V), but this effect is not always shown and it is doubtfully significant.

TABLE V

Effect of Different Methods of Sterilization on Enzymic Activity and Fungal Growth

Method of sterilization	Colour after sterilization	Init. pH	Final pH	R.T. in mins.	Dry wt. of myc.
Filtration	No change	8·0	8·0	75	1·8
Steaming	Light yellow	7·8	8·4	25	2·0
Autoclaving at 5 lb.	Yellow	7·8	8·3	25	2·2
,, 10 lb.	Dark yellow	7·8	8·3	25	2·5
,, 15 lb.	Light brown	7·5	8·2	25	1·9
,, 20 lb.	Brown	7·4	7·5	35	1·6

The beneficial effect is obtained only when the carbohydrate (glucose) is heated with the phosphate; the presence of the other constituents is immaterial. Tribasic and dibasic phosphates are effective, but not monobasic. Good results are also obtained with dilute NaOH and Na_2CO_3, though so far somewhat inferior to those with alkaline phosphate. Further work may show that the effect depends essentially upon the maintenance during heating of a suitably alkaline pH.

TABLE VI

Effect of Decolorization on Enzyme Production and Mycelial Growth

Medium	Init. pH	Final pH	R.T. (mins.)	Dry wt. of myc.
A	8·0	7·9	90	1·8
B	7·4	8·2	25	2·1
C	7·4	8·2	30	1·6

As was found by Fulmer, Williams, and Werkman (loc. cit.) in their study of bacterial growth substances, the yellow-brown colour which is associated with high production of enzyme is merely incidental. This is illustrated in Table VI, in which the standard medium (with 3 per cent. glucose and 0·1 per cent. NaCl) was set up in the following three ways, the + signs indicating that the constituents so separated were autoclaved separately:

A. $MgSO_4$+K_3PO_4+rest of medium; no colour produced.

B. $MgSO_4$+rest of medium; light brown colour.

C. As in B, and then decolorized by adding 2 g. active charcoal per 100 ml. of medium and shaking for one hour. The medium was then passed through a sterile Seitz filter which also removed the charcoal.

The somewhat reduced growth and enzymic activity in C, as compared

with B, may have been due to adsorption of some nutrient components by the charcoal. Nevertheless the main result is clear, viz. that the coloured and then decolorized medium C is much superior to the colourless medium A.

Damle suggested that the stimulative effect obtained by heating glucose with alkaline phosphate might be due to formation of a glucose phosphate. The fact, however, that most if not all of the beneficial effect can be obtained by heating the sugar with dilute sodium carbonate or hydroxide disproves that view. Further evidence on this point was obtained chromatographically. A solution containing 7·5 per cent. glucose and 0·34 per cent. K_3PO_4 was autoclaved for 20 minutes at 15 lb. pressure, and after chromatographic

TABLE VII

Effect of Caramel Solution on Enzyme Production and Mycelial Growth

Medium	Init. pH	Final pH	R.T. (mins.)	Dry wt. of myc.
A	8·1	8·1	65	2·1
B	7·5	8·0	30	2·0
C	7·9	8·0	25	2·5
D	8·0	8·0	25	2·2

separation on paper, tested for phosphoric esters by the method of Bandurski and Axelrod (1951). Solutions of glucose-1-phosphate and 3-phosphoglycerate were used for purpose of reference. No spots indicative of glucose phosphate esters were found in the chromatogram of the autoclaved solution.

The development of a yellow-brown colour on heating glucose with alkaline phosphate is presumably due to a certain amount of caramelization taking place. It was interesting therefore to test the effect of glucose which has been caramelized by dry heat. Five grams of glucose were heated in an oven at 150–5° C. for 20 minutes and the brown mass after cooling taken up in 10 ml. water giving a '50 per cent. caramel solution'. When this solution was added to the culture medium, enzyme production was strongly enhanced as shown in Table VII. The standard medium was set up in the following ways, with the same notation as for Table VI:

A, $MgSO_4$+K_3PO_4+rest of medium.
B, $MgSO_4$+rest of medium.
C, as in A, but with 0·25 per cent. of 'caramel solution' added.
D, as in C, but medium decolorized in the manner already described.

The active substance is presumably therefore a breakdown product of glucose, the accompanying colour change being of no consequence.

In eight experiments in which a comparison was made between treatments A, B, and C of Table VII the following data were obtained:

Medium A	Av. R.T. 74±4·6 min.
„ B	„ „ 29±1·5
„ C (with 0·33% caramel)	„ „ 28±9·6

Statistical analysis: A–B and A–C, $P < 0{\cdot}01$

These results were confirmed by the viscometric method, as shown in Fig. 3.

Glucose which had been heated in alkaline solution and dry-heated glucose were chromatographed on paper, with a 4:1:5 mixture of n-butanol, acetic acid, and water. The spots were located with a benzidine reagent (0·5 g.

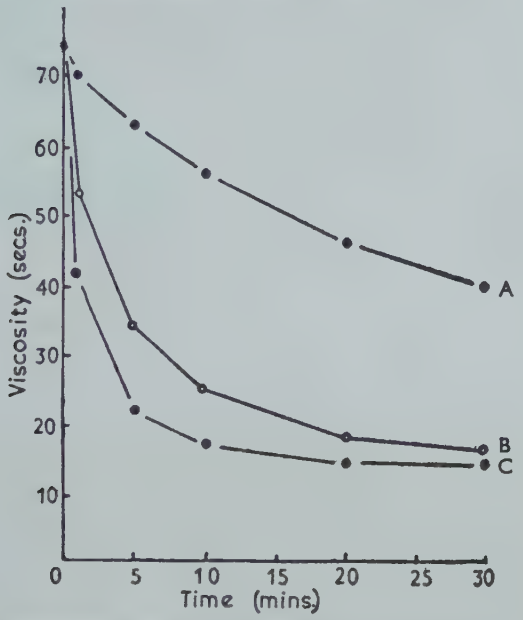

FIG. 3. Effect of caramelized glucose and method of autoclaving on production of pectolytic enzyme by *P. de Baryanum.*
For preparation of media A, B, and C, see text (preceding page).

benzidine, 10 ml. acetic acid, 10 ml. 45 per cent. trichloracetic acid, 10 ml. 95 per cent. ethanol). Various sugars and breakdown products of these were similarly treated; the results are shown in Fig. 4.

From Fig. 4 it may be concluded that some fructose or mannose and possibly glyceraldehyde and dihydroxyacetone are formed by autoclaving glucose in the presence of K_3PO_4 or NaOH or by heating dry glucose at 150–5° C. for 20 minutes. The last treatment also produced a number of substances which moved more slowly than glucose on the chromatogram.

The effect of mannose and fructose, separately or together, as partial replacements of glucose in the standard medium is shown in Table VIII. Except in the first medium, the sugars were autoclaved separately, as indicated by the + signs. The following media were set up:

A. $MgSO_4$+rest of medium
B. „ +3% glucose+rest of medium
C. „ +2% „ + „ „ +1% mannose
D. „ + „ „ + „ „ +1% fructose
E. „ +1% „ + „ „ + „ „ +1% mannose.

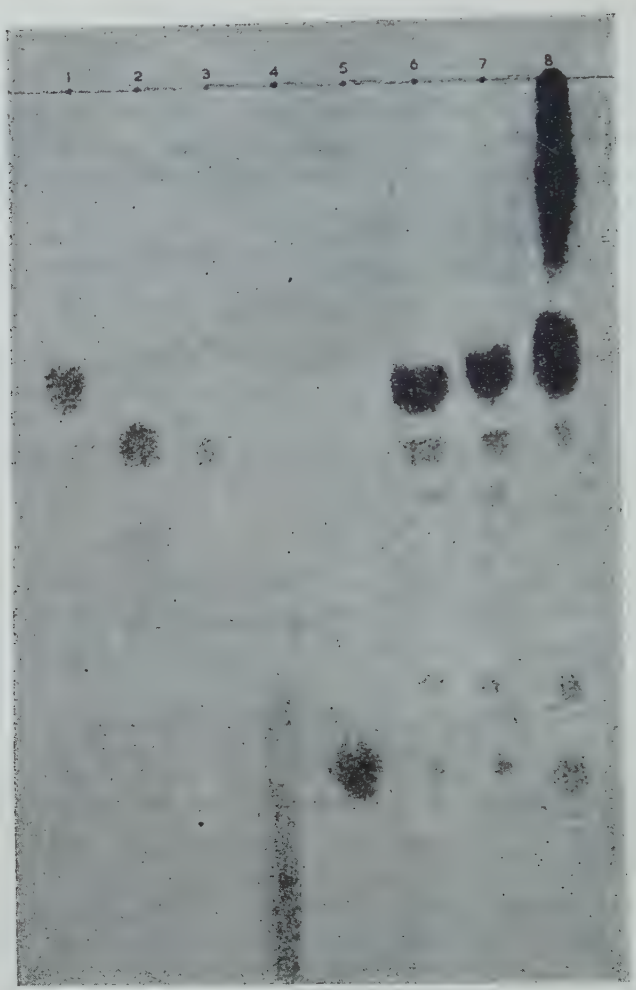

FIG. 4. Chromatographic separation of sugars and derived compounds.

Spots: 1, glucose; 2, mannose; 3, fructose; 4, glyceraldehyde; 5, dihydroxyacetone; 6, glucose autoclaved with K_3PO_4 solution; 7, glucose autoclaved with NaOH solution; 8, caramelized glucose.

TABLE VIII

Effect of Mannose and Fructose on Enzyme Production and Mycelial Growth

Medium	Init. pH	Final pH	R.T. (mins.)	Dry wt. of myc.
A	7·5	8·2	25	2·1
B	8·0	8·2	70	2·0
C	8·0	8·2	60	1·8
D	8·0	8·2	60	1·9
E	7·9	8·2	50	2·2

From the above results it was concluded that though a mixture of glucose and mannose, glucose and fructose, or glucose, fructose, and mannose was

slightly better than glucose alone, all were inferior to glucose autoclaved with alkaline phosphate.

Glyceraldehyde and dihydroxyacetone were similarly tested in a series of media as follows, the notation being the same as for the preceding three Tables.

A. $MgSO_4$+rest of medium.
B. „ +K_3PO_4+rest of medium.
C. „ + „ + „ „ +0·1% fructose
D. „ + „ + „ „ +0·1% glyceraldehyde
E. „ + „ + „ „ +0·1% dihydroxyacetone
F. „ + „ + „ „ + „ „ +0·1% glyceraldehyde
G. „ + „ + „ „ + „ „ + „ +0·1% fructose
H. „ + „ + „ „ +0·33% caramel solution.

The results are shown in Table IX.

TABLE IX

Effect of Fructose and Certain Trioses on Enzyme Production

Medium	Init. pH	Final pH	R.T. in mins
A	7·5	8·1	30
B	8·0	8·2	65
C	„	„	50
D	„	„	30
E	„	„	45
F	„	„	33
G	„	„	37
H	„	„	25

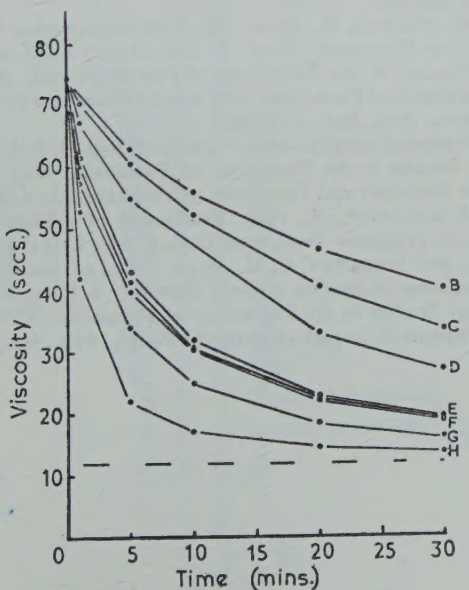

FIG. 5. Effect on pectolytic enzyme production of *P. de Baryanum* by addition of various substances to the standard medium. For composition of media A-H, see text (curve for A lies between curves for E and F). The interrupted horizontal line indicates the viscosity of water.

The enzymic activity of media A–H of Table IX was also tested by viscometry and results are illustrated in Fig. 5.

Table IX and Fig. 5 show that the addition of glyceraldehyde to the medium considerably stimulated enzyme secretion. In the chromatogram of caramel (Fig. 4) there were indications of fructose and dihydroxyacetone in addition to glyceraldehyde. But when a mixture of glyceraldehyde, dihydroxyacetone, and fructose was used, it proved to be inferior to glyceraldehyde alone. However, the possibility of more active enzyme production by using appropriate concentrations of these three substances still remains. The main evidence at the moment is that glyceraldehyde is the breakdown product which is most active in stimulating enzyme production.

Acknowledgements

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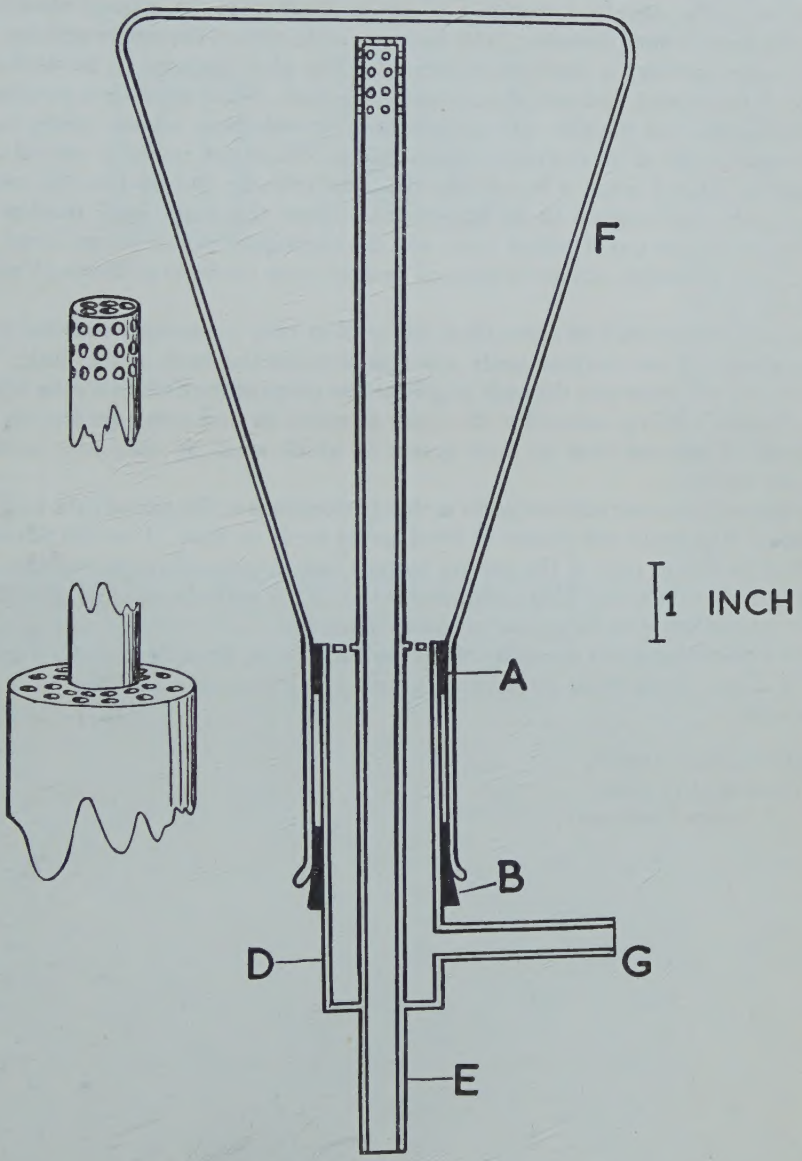
NOTE

An Efficient Aerating Washer for Seeds or Tissue Discs

Most laboratories of plant physiology find it necessary from time to time to subject plant materials (e.g. seeds or tissue discs) to prolonged washing or soaking in a stream of water under conditions of good aeration. In the writer's laboratory this need has been met over the years by a sequence of devices which have evolved



under the selective influence of floods and other minor mishaps. The object of this note represents the simplest and yet most efficient design so far achieved, and it is hoped that it may be of interest to other departments of botany.

F is a conical flask of 1 litre capacity into the neck of which the metal water-circulator is fixed by two rubber rings A and B. A is of such a size that it just slides up into the neck of the flask without compression, while B is slightly larger and tapered so that it makes a rigid joint. The circulator itself is made of metal (copper or brass is most convenient) and consists of two concentric cylinders D and E. The top of the inner cylinder E is sealed off but perforated (in the manner shown) by holes of about 2 mm. diameter. The bottom end is open. The upper annular end of the outer cylinder is similarly perforated. The plant material to be washed is placed in the conical flask and the circulator inserted. The flask is then inverted as in the diagram and the side inlet G connected by wide bore rubber tubing to the *bottom* outlet end of an ordinary suction pump. When the pump is turned on a mixture of air and water is forced into the outer cylinder and up into the conical flask via the perforations in its upper end. When the water level reaches the perforations at the top of tube E water and the accompanying air escape down this tube. Thus a constant stream of agitated aerated water can be kept flowing through the flask.

Buoyant objects such as tissue discs will tend to keep in constant rotation in the water stream. Less buoyant seeds will sink towards the neck of the flask. The efficiency of the apparatus depends largely on the shape of the flask, since the conical sides direct all falling material to the point at which air and water are issuing into the flask. There are thus no dead spaces in which seeds or discs may settle in stagnant water.

An important constructional point is that perforations at the top of tube E should be adequate to avoid any chance of blockage by seeds or discs. It is also advisable to attach to the *air inlet* of the suction pump a long portion of rubber tubing with its open end in the sink. This avoids floods should the unlikely accident of a blockage occur and water be forced out of this air inlet.

The writer wishes to express his thanks to Messrs. N. Brownbridge, C. Funnell, and S. White whose ideas contributed so materially to the successful evolution of the washer.

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